# THE PHYSIOLOGY AND BIOCHEMISTRY OF LACTATION

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BY

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## PREFACE TO THE ENGLISH EDITION

In preparing an English version of my book *Recherches Recentes sur la Physiologie et la Biochimie de la Sécretion Lactee* which was published in December 1954 as No 18 of the series *Actualites Biochimiques* under the imprint of Editions Desoer of Liege and Masson et Cie of Paris, I have brought the text up to date by inserting brief references to papers published since that time which appear to be important Apart from this and the correction of such errors as have been brought to my attention the text remains substantially unchanged In addition to the acknowledgements made previously I should like to express my thanks to Mrs Gwen Stephen of the Classics Department University of Reading for information about the mythology of the origin of the galaxy

After completion of the manuscript unfortunate circumstances beyond my control intervened to prevent me from seeing this book through the press This arduous and exacting task was at once willingly undertaken by my colleague, Dr A T Cowie to whom my grateful thanks are due Without his help, unstintingly given, this book could never have been published I am also grateful to Miss Anne Pendree and Mrs M C Lloyd for help in the preparation of the index

The subject matter of these lectures embraces a field which has occupied the Physiology Department of the National Institute for Research in Dairying for many years and in offering them in the form of a book I hope that it will be found useful by medical and veterinary students particularly those taking the degree of B Sc in Physiology or Biochemistry

*Shinfield June 1955* S J FOLLEY

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Lister Institute, London as well as to Dr Raymond Michel of the Collège de France who also helped me considerably in various ways with the preparation of my lectures

The expenses entailed in connection with the translation and preparation of these lectures for publication were met by grants from the Bureau Scientifique de l Ambassade de France in London, through the courtesy of Monsieur Rene Varin, *Conseiller Culturel and Miss A M Vidal Hall and by the* Ciba Foundation through the courtesy of the Director Dr G E W Wolstenholme O B E To both these bodies I express my sincere thanks

I would also like to acknowledge the great debt I owe to my collaborators whose work forms the basis of the greater part of these lectures and who kindly gave me permission to quote their unpublished experiments Their names will be found in appropriate places in the text

It is a pleasure also to thank Mr G Machin for drawing the figures and Mr N Gruber for skilled photographic work and my secretary Mrs Jean King My thanks are also due to all who have given me permission to reproduce material from published papers

S J FOLLEY

# FOREWORD

The lectures which comprise this book were delivered at the College de France, where I was invited as a visiting Professor in December, 1953 In preparing them for publication I have changed them as little as possible from their original form

I should like first to express to Monsieur l Administrateur, M Edmond Faral, and the Professors of the Collège de France my deep appreciation of the honour accorded to me in issuing their invitation My gratitude is especially due to Professor Jean Roche who gave me the hospitality of his Department during my stay it is to him that I owe the suggestion that these lectures might be published I am greatly in his debt for his invaluable assistance in preparing this edition for publication in the series *Actualites Biochimiques* I should also like to record my indebtedness to Professor Robert Courrier, Membre de l'Institut who not only introduced me to my audience but in various ways did much to make my stay in Paris pleasant

There would have been no question of my being in a position to accept the invitation to lecture in the College de France had I not been able to enlist the moral support and encouragement of Professor A G Lehmann of the Department of French Studies, University of Reading who kindly put at my disposal the valuable help of two of the staff of his Department Monsieur Jean Gautier who willingly undertook the difficult task of translating my lectures into French and Mrs Antoinette Hunt, who took endless trouble to improve my French pronunciation I would like to express my sincere gratitude to them

For help with the translation of technical terms not found in dictionaries my best thanks are due to Professor Marc Klein of the University of Strasbourg, and Dr L Sazbo then of the

# CHAPTER I

# RECENT STUDIES ON THE DEVELOPMENT OF THE MAMMARY GLAND

## NORMAL POST NATAL DEVELOPMENT

Earlier comparative studies on the normal development of the mammary gland in different reproductive phases, which laid the foundations of our knowledge of the endocrine control of mammary development, suggested that growth of the mammary duct system depended upon the presence of oestrogen and that for extensive lobule alveolar development luteal influence was necessary in addition Later, when experimental analysis of the hormonal control of mammary growth by experiments involving the administration of pure hormones to spayed females or males became possible, these generalities resulting from observational studies though substantiated in broad outline were found to be over simplifications in the case of some species The reader is referred to the review by Folley (1952) for an account of these observational and experimental studies

Recently there has been a quickening of interest in the development of quantitative and objective methods for studying mammary growth One reason for this is the prospect of practical application of fundamental knowledge of the hormonal control of the mammary gland to the artificial stimulation of udder growth and lactation in dairy cattle Such techniques should also prove valuable to workers in the cancer field who are interested in the influences determining the architecture of the mammary gland in various strains of mouse The subject has reached a stage of development at which the subjective and qualitative methods that have served in the past can no longer be expected to lead to further progress the extension of knowledge demands the application of quantitative methods

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and mouse (Flux, 1954a) The whole mount technique is of course not applicable to 'three dimensional glands like the udder of the ruminant the human breast or the mamma of the guinea pig, and other methods must be used for these Mr K C Richardson of the Department of Anatomy, University College London with whom we have had the advantage of working in collaboration, has evolved a technique for the estimation of the total alveolar surface area in the goat udder based on a method originally developed by another worker for application to lung tissue Figure I 1 shows the excellent linear relationship between total alveolar area and milk yield in a series of hormonally developed goat half udders (Richardson 1953), thus illustrating the usefulness of Richardson s technique This technique, since it also affords an estimate of the area of the secretory epithelium per unit volume of tissue ( 'porosity index ) also gives a measure of the mean diameter of the alveoli This type of measurement as applied to the goat udder, has been further developed by Benson Cowie, Cox Flux and Folley (1955)

Though it has long been known that in many species the mammary duct system undergoes a slow extension during the period from birth to puberty it has generally been believed that mammary growth remains substantially in abeyance until puberty when the beginning of ovarian cycles was thought to initiate a phase of a rapid mammary development (see review by Folley, 1952) Quantitative studies of the rate of increase in the mammary gland area in relation to the rate of enlargement of the body surface show that this is not the case for the rat and mouse Nor as was shown some years ago, is it true for the monkey It probably does not hold for any mammal

The first use of relative growth analysis which was developed in France by G Teissier and in England by J S Huxley, for the quantitative study of mammary growth in the normal female was made many years ago in the monkey by Folley, Guthkelch and Zuckerman (1939) Table I 1 explains the principles of relative growth analysis and gives the terminology proposed by Huxley and Teissier (1936) The value of the equilibrium constant $\alpha$, which can be evaluated by a simple graphical method indicates whether the organ

In species, such as the mouse, rat and rhesus monkey, in which the mammae, save in late pregnancy and lactation, may for practical purposes be regarded as more or less flat sheets of tissue so that they can be prepared for histological study as whole mounts, changes in the mammary gland area, that is the area bounded by a line joining the periphery of the ducts give a measure of the rate of general extension of the duct tree These measurements of course provide no information about morphological changes within that area such as increased branching of the duct system or the formation or multiplication of alveoli In 1947 Dr A T Cowie and I attempted to devise a scoring system, semi quantitative and susceptible of statistical analysis, which we hoped would afford some measure of qualitative changes of this nature (Cowie & Folley, 1947) More recently, improved and more objective techniques for estimating the degree of branching have been developed in our laboratory for the rat (Silver, 1953a)

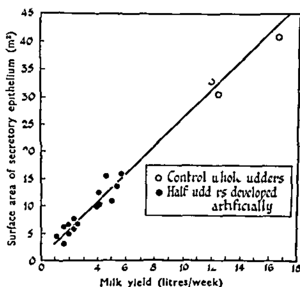



Fig I 1 Relation between milk production and total alveolar area in a series of half udders from goats (*from Richardson 1953*)

showed that over a wide range of body weights the breast in the non pregnant female macaque grows *faster than the body* according to the simple allometric law – presumably under the specific influence of ovarian hormones More detailed studies of the dynamics of mammary growth using relative growth analysis were later made in our laboratory in the rat (*Cowie, 1949*) As *Figure I 2 shows* Dr Cowie found that in the female rat the total area of all the mammary glands increased at approximately the same rate as the body surface, that is isometrically, until the 22nd–23rd day when a phase of rapid allometric growth abruptly set in In this phase α had a value of 3 03 which means that the mammae were increasing in area about three times faster than the body surface Ovariectomy at the 22nd day prevented the onset of the allometric phase and isometric growth (α not significantly different from unity) continued Since in our strain of rat the vagina does not open nor oestrous cycles begin until the 35th–42nd day *it is clear that the mammary gland begins to grow faster than* the body well before puberty a result which probably would not have been revealed merely by the conventional qualitative examination of whole mounts or thin sections In the immature male rat, the increase of mammary area on body surface was very slightly but significantly allometric since α

TABLE I 2

*Equilibrium constants for mammary growth relative to body growth in the Norway rat*

(from Silver, 1953*a*)

| Group | Equilibrium constant (α) | | Significance of the difference |
|---|---|---|---|
| | (a) Silver (1953*a*) | (b) Cowie (1949) | (a) — (b) |
| Females (intact) | 3 40±0 17 | 3 03±0 13 | P = 0 09 |
| Males (intact) | 1 17±0 05 | 1 11±0 03 | P > 0 1 |
| Females (ovariectomized) | 1 45±0 09 | 1 05±0 10 | P = 0 005 |
| Males (castrated) | 1 34±0 08 | 1 06±0 12 | P = 0 07 |

TABLE I 1

*Relative growth analysis*

Law of simple allometric growth (Huxley, Teissier)

$y = bx^{\alpha}$

or $\log y = \alpha \log x + \log b$

$y$ = total mammary gland area

$x$ = area of reference standard (body)

$\alpha$ = equilibrium constant

$b$ = constant

If the law is obeyed, a plot of $\log y$ against $\log x$ gives a straight line with the slope $\alpha$

$\alpha > 1$ positive allometry

$\alpha < 1$ negative allometry

$\alpha = 1$ isometry

under study is growing faster than the body as a whole, if it is then $\alpha$ is greater than unity In our study of the monkey, we

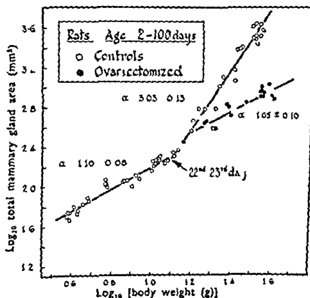



Fig I 2 Relation between total mammary gland area and (body weight) $^{\alpha}$ in the female rat *(from Cowie 1949)*

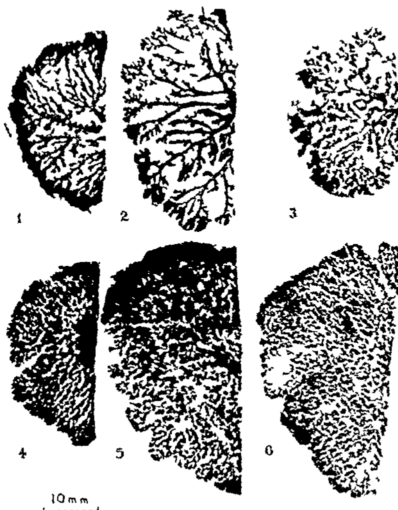
Evidence bearing on this question has recently been obtained in our laboratory (Silver, 1953*b*) Mrs Silver found that the mammary gland of the suckling rat shows no growth response to physiological doses of oestrogen administered between the 11th and 19th days of age a finding which is in conflict with the earlier results of Astwood, Geschickter and Rausch (1937) who reported that oestrogen would begin to evoke growth of the rat mammary gland as early as the 16th day However, it should be noted that the doses used by Astwood and his colleagues were relatively high and probably unphysiological It was further found that low doses of oestrogen administered together with an anterior pituitary extract, ineffective by itself caused appreciable growth of the mammae in the young suckling rat Similarly Sykes and Wrenn (1950) have found that the mammary growth promoting effect of oestrogen and progesterone in young calves was potentiated by anterior pituitary extract If, therefore as has been suggested the role of the anterior pituitary in mammary growth is to sensitize the mammae to the mammogenic effect of oestrogen it would appear that in the suckling rat the pituitary is not yet fully functional in this respect These experiments however do not exclude the possibility that in the rat at least some factor ingested in the milk perhaps androgen, is responsible for the failure of the pituitary to play its part in mammary growth

Relative growth analysis has also added to our knowledge of the factors governing the growth of the mamma in the normal male rat The fact that the gland of the male rat grows isometrically or nearly so, and that its specific growth rate is not affected by castration demonstrates more decisively than had previously been possible that the testis has very little effect on the growth of the mammary duct system in the intact male rat However castration at 21 days prevents for a time the development of the lobules of alveoli which constitute a remarkable and characteristic feature of the mammary gland in normal male rats first described long ago by Turner and Schultze (1931) Alveolar development in the mamma of the male rat would thus seem to depend on the presence of the testis Nevertheless, as we showed some years ago, (Cowie and Folley, 1947) some alveoli eventually develop in the mammae of immaturely

had a value of 1 1 and the relative growth rate was not significantly altered by castration at 22 days These findings have since been confirmed and extended in our laboratory with the same strain of rat by Mrs Marian Silver She found, however, as shown in Table I 2 which gives her equilibrium constants together with those of Dr Cowie, that ovariectomy at ten days of age, that is eleven days earlier than in the experiments of Cowie, was eventually followed by a phase of slightly allometric growth of the mammae (Silver 1953*a*) This might be due to the action of mammogenic steroids secreted by the adrenal cortices under unremitting stimulation by a pituitary released from ovarian control

Similar studies carried out in our laboratory by Dr D S Flux on female mice of Strong s CHI strain showed that here too the mammae grew isometrically over the 7th–21st days of life As in the rat, a phase of marked allometry, the onset of which could be prevented by ovariectomy, set in at about 24 days The value obtained by Dr Flux for the equilibrium constant during the allometric phase ($\alpha = 5\ 22$) indicates that after weaning the mouse mammary gland grows about five times faster than the body surface by contrast with that of the rat which grows only three times faster than the body (Flux 1954*a*) Whether this species difference in specific growth rate is in any way connected with the relative basal metabolic rates of the two forms is a matter for speculation In this strain of mouse the phase of rapid mammary duct growth does not precede the onset of cyclic ovarian activity, which occurs at about 28 days, by so long an interval as in our strain of rat

While these studies establish the necessity of the ovary for the abrupt change from isometric to allometric mammary growth the nature of the mechanism involved and the reason why allometric growth begins just when it does are not known with certainty It is possible to discuss this question in terms of the time of maturation of pituitary gonadotrophic function and of ovarian responsiveness to pituitary influence Nevertheless the fact that the change from isometry to allometry closely follows weaning may not be coincidental and raises the question whether the female rat secretes in her milk a substance which antagonises the mammogenic action of oestrogen




The figures represent whole mounts of approximately half a mammary gland from a male rabbit (*from Scharf and Lyons* 1941)

1 30 i u oestrone per day
2 240
3 960

4 30 i u oestrone — 1 mg progesterone per day
5 240
6 960

gonadectomized rats and just as in the case of the female spayed at 10 days already referred to, it seems reasonable to seek the cause in enhanced production by the adrenal cortex of mammogenic steroids, such as androgens or perhaps progesterone, due to the disturbance in the endocrine balance brought about by gonadectomy

EXPERIMENTAL ANALYSIS OF HORMONAL INFLUENCES

*Ovarian hormones* We now come to the experimental analysis of hormonal influences in mammary growth and will consider first the ovarian hormones Early work involving hormone administration pointed to the general conclusion that of the ovarian hormones oestrogen is more particularly responsible for the growth of the mammary duct system while progesterone, acting in concert with oestrogen, is necessary for full alveolar growth Later work has shown that in forms such as the mouse, rat and monkey progesterone alone, if enough is given, will evoke development of the alveoli and perhaps of the ducts in spayed animals unprimed with oestrogen For a review of the above mentioned studies the reader should consult Folley (1952)

The general conclusion emerging from the early studies a conclusion which incidentally remains unshaken by the latest work, was that both oestrogen and progesterone are needed for the experimental growth of a gland comparable morphologically with that of mid pregnancy However it was apparent from the outset that various species show considerable differences in the nature of the growth response of the mammary rudiment to oestrogen and on this basis three broad categories among laboratory and domestic animals can be distinguished though we must not forget that interpretation may be complicated by the possibility that spayed animals may still possess a source of progesterone in the adrenal cortex

The first category includes the mouse rat, rabbit and cat in all of which the male gland is equipotential with that of the female In the mammae of spayed females or of intact or castrated males of this group oestrogen in physiological doses evokes primarily and mainly duct growth, alveoli only appearing when higher doses are given and when administration is prolonged This last point is illustrated by the rabbit in which,

as Lewis and Turner (1941) and others have shown, mammary alveoli can be developed by high doses of oestrogen if applied for long enough It might therefore be questioned whether the rabbit should be included in the present category However, the striking synergism between oestrogen and progesterone shown in the beautifully clear-cut results of Lyons and his collaborators (Lyons and McGinty 1941, Scharf and Lyons, 1941), some of which are shown in Plate 1 3 effectively disposes of such doubts This Plate shows whole mounts of mammary glands of male rabbits some of which had received oestrone and others oestrone and progesterone The synergistic action of progesterone in causing lobule alveolar growth is quite evident.

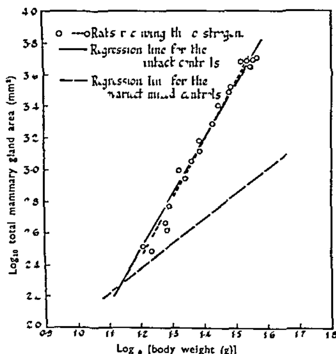



Fig 1 4. Relation between the total mammary gland area and (body weight)$^{x}$ in the ovariectomized rat receiving a dose of oestradiol dipropionate which was increased stepwise with the body weight *(from Silver 1953a)*

normal mammary growth process as judged by the equilibrium constant and by the increase in duct buds and branches by starting with a dose of 0 1 μg oestradiol dipropionate every other day when the rats were 21 days old and increasing the dose stepwise with the body weight Figure I 4 shows how closely she was able to imitate the relative mammary growth rate of the intact female rat Mrs Silver was also able to obtain evidence that as R Hertz first showed for the oviduct of the fowl and for the rat uterus folic acid is concerned in some way in the growth response of the mammary ducts to oestrogen This is illustrated in Figure I 5 which shows that the relative rate of mammary growth in response to the administration of oestradiol dipropionate is diminished by the administration of aminopterin, a powerful folic acid antagonist, even when the food intake of the control rats was restricted to that of those receiving the antagonist (Silver, 1954)

TABLE I 3

*Effect of oestrone on the area of the mammary glands of CHI mice*

(from Flux 1954*a*)

6 ovariectomized mice per group

| *Treatment daily injections from 21—42 days of age* | *Total mammary gland area area at 42 days of age* mm² |
|---|---|
| 0 1 ml arachis oil | 64 |
| 0 01 μg oestrone in 0 1 ml oil | 129 |
| 0 055 μg oestrone in 0 1 ml oil | 669 |
| 0 10 μg oestrone in 0 1 ml oil | 675 |
| Intact controls | 565 |

In the mouse Dr Flux has found in our laboratory as shown in Table I 3 that 0 01 μg oestrone daily from 21 to 42 days of age about doubled the total mammary area over that of ovariectomized controls at 42 days so that the threshold dose for the mouse must be somewhat lower than this (Flux, 1954*a*) However, somewhat higher doses 0 055 μg/day, were

Information about the body levels of oestrogen which probably obtain during the allometric phase of mammary growth in the immature normal female rat has been obtained by Mrs Silver in our laboratory by means of relative growth analysis She found (Silver, 1953*a*) the threshold dose of oestrogen for the induction of allometric mammary growth in spayed immature female rats to be in the neighbourhood of 0 05µg oestradiol dipropionate injected subcutaneously every two days In the young spayed rat she could best mimic the

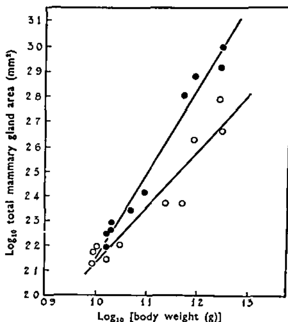
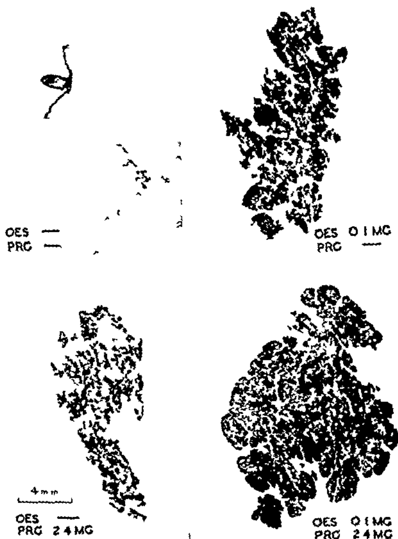



Fig I 5 Effect of aminopterin on the growth of the mammary gland in the rat (*from Silver 1954*)

| Group | Equilibrium constant α |
|---|---|
| o Aminopterin + oestradiol dipropionate | 2 29 ± 0 08 |
| | p 0 001 |
| • Oestradiol dipropionate alone (pair fed controls) | 3 45 ± 0 06 |




Sections at the same magnification of mammary glands from ovariectomized virgin guinea pigs which had received daily doses as indicated for 68 days of oestrone (OES) progesterone (PRG) or oestrone+progesterone (*photograph provided by Dr A T Cowie*)

necessary to attain mammary areas not significantly different from those of comparable intact mice

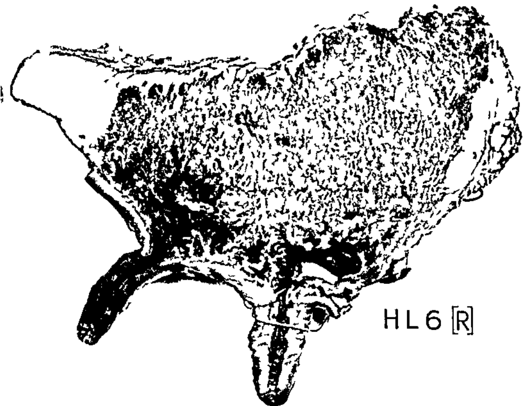
The second of the aforementioned categories comprises forms in which oestrogen in more or less physiological doses evokes extensive growth of the mammary lobule alveolar system as well as of the duct tree The earliest known example is the guinea pig in which, as is well known, functional mammae can readily be developed in either sex simply by oestrogen administration Though earlier workers believed that combined treatment with oestrogen and progesterone had no greater effect on the guinea pig mamma than treatment with oestrogen alone recent work by Dr Cowie, who gave the two hormones in various ratios to spayed females and submitted serial sections of the mammae to a semi quantitative system of gland assessment indicates that both progesterone and oestrogen in the correct ratio are necessary for optimal lobule alveolar development in the guinea pig Some of his results (Cowie 1951) are illustrated in Plate I 6 which shows typical sections from the mammae of guinea pigs receiving the hormone treatments indicated The effect of progesterone on lobule alveolar growth can be seen by comparing the upper and lower right hand figures

Also included in this category are the domestic ruminants, in particular the cow and goat in which however the male mammary rudiment is not equipotential with that of the female The extensive udder development obtainable with oestrogen alone was first demonstrated in the intact goat in our laboratory fifteen years ago (Folley Scott Watson and Bottomley, 1940, 1941) and similar results were later obtained by numerous workers in extensive experiments on cattle

In most of the earlier investigations no detailed morphological studies were made, the occurrence of extensive alveolar growth in response to oestrogen being inferred from the considerable milk yields often given by the treated animals and sometimes from macroscopic inspection of sliced udders (Plate I 7) Moreover, since intact animals were mostly used the possibility that progesterone of ovarian origin participated in the responses could not be discounted Nevertheless the magnitude of the milk yields, the appearance of macro udder slices from the treated animals, (e g Plate I 7) and such

histological observations as those of Lewis and Turner (1942) and Mixner and Turner (1943) on oestrogen treated goats, left no doubt that treatment with oestrogen alone caused extensive lobule alveolar development in the ruminant That this conclusion was justified is shown by our later experiments on gonadectomized goats, one of which is illustrated in Plate I 8 which shows a section of a half udder prepared by Mr K C Richardson from an ovariectomized virgin goat which was treated with oestrogen for 20 weeks extensive alveolar development occurred in this gland

However the great variability of the lactational responses to a given treatment observed by most workers, and the fact that even the best milk yields were below those to be expected from similar animals after parturition, suggested that udders artificially developed by oestrogen treatment tend to exhibit some abnormality or deficiency in glandular structure Indeed, over ten years ago Mixner and Turner (1943) reported histological abnormalities in the glands of virgin goats treated for long periods with stilboestrol These mainly comprised grossly dilated or cystic alveoli with papillomatous epithelial outgrowths Combinations of oestrogen and progesterone, on the other hand gave glands in which cystic alveoli were not so prominent Recently our group (Cowie, Folley, Malpress and Richardson 1952) have made extensive morphological studies, by the aforementioned special methods developed for the purpose by Mr Richardson, of udders grown in immaturely ovariectomized virgin goats by treatment with combinations of hexoestrol and progesterone in various proportions and at different dose levels in comparison with the udders resulting from treatment with hexoestrol alone The latter showed various histological abnormalities of which the most striking and probably most significant functionally was a marked deficiency of total epithelial surface probably because most of the alveoli were too large as compared with those found in the udders of normally lactating goats A section showing such dilated alveoli is shown at the bottom of Plate I 9 The middle section shows that they are largely absent from udders grown with suitable combinations of oestrogen and progesterone, both these may be compared with the top section which is from a normal goat udder All are photograph




Section of the udder developed by treatment with hexoestrol from a heifer (From Folley and *Malpress* 1944)

indication which was upheld by morphological studies of these udders

Sykes and Wrenn (1951) have described somewhat similar histological abnormalities in the artificially grown udders of calves and cows receiving stilboestrol and found that progesterone in sufficient doses largely abolishes these abnormal manifestations Moreover, Reineke Meites Cairy and Huffman (1952) have succeeded in developing in heifers and cows by treatment with oestrogen and progesterone udders which, because they have yielded much more milk than has been obtained in previous studies involving treatment with oestrogen alone must presumably be more comparable structurally with the udder resulting from normal pregnancy

The last of our three categories of animals comprises those in which oestrogen alone in physiological doses causes little or no mammary development not even of ducts The only instance which has been adequately reported is the bitch which was studied by Trentin de Vsta and Gardner (1952), but since Hammond and Marshall (1930) found very little mammary duct growth in the oestrous ferret even after prolonged oestrus it seems likely that the ferret belongs to this class also

*Anterior pituitary hormones* Let us now turn to the consideration of the role of anterior pituitary hormones in mammary growth The relation of the anterior pituitary to mammary development became a live issue soon after the discovery by Stricker & Grueter (1928) in Strasbourg of the lactogenic action of anterior pituitary extracts The early report of Corner (1930) that anterior pituitary extracts developed the mammae of spayed rabbits was the forerunner of a number of similar claims For instance W R Lyons has always maintained that lactogenic pituitary preparations would cause mammary development and his ingenious demonstration (Lyons 1942) that purified prolactin will cause localized alveolar hyperplasia in those and only those sectors of the rabbit mammary gland into which minute quantities of the hormone solution have been injected would seem to afford good evidence (provided the prolactin used was of high purity) that at least one anterior pituitary hormone can exert direct mammogenic effects at any rate on a gland which is under the influence of a normal pituitary

ed at the same magnification Another of these histological abnormalities, an immature lobule, is shown in Plate I 10, and Plate I 11 shows the epithelial papillomata which were also found Table I 4 gives results which show that progesterone prevents the occurrence of all three types of abnormal develop ment provided the oestrogen dosage is not excessive Richard son (1953) has since confirmed, on further examination of

TABLE I 4

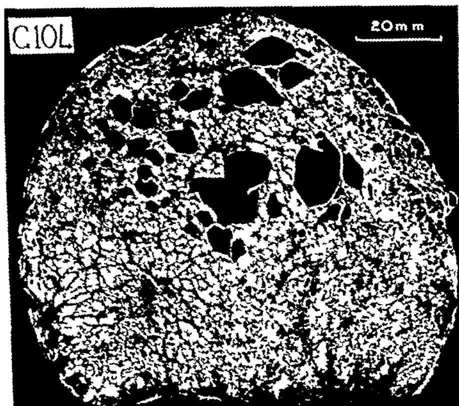
*Occurrence of various types of histological abnormality in hormonally developed goat udders*

(from Cowie, Folley, Malpress and Richardson, 1952)

| Treatment | No of goats | No of goats in which the udder showed | | | | |
|---|---|---|---|---|---|---|
| | | Cystic alveoli | | Epithelial puckering | | Immature lobules |
| | | Sub-capsular | Intralobular | Alveoli | Ducts | |
| Hexoestrol (1 mg/day) | 7 | 5 | 1 | 0 | 3 | 1 |
| Hexoestrol (1 mg/day) + progesterone (40 mg/day) | 6 | 6 | 2 | 4 | 4 | 2 |
| Hexoestrol (0 25 mg/day) | 2 | 2 | 1 | 2 | 2 | 2 |
| Hexoestrol (0 25 mg/day) + progesterone (100 mg/day) | 2 | 0 | 0 | 0 | 0 | 0 |
| Hexoestrol (0 25 mg/day) + progesterone (40 mg/day) | 2 | 1 | 0 | 0 | 0 | 0 |

material from these experiments that progesterone significantly increased the area of secretory epithelium per unit volume, that is it produced alveoli falling within the normal size range More recent experiments in our laboratory (Benson *et al* 1955) indicate that suitable doses of oestrogen and progesterone given to spayed virgin goats develop udders which give reasonably uniform milk yields as between differ ent animals while the incidence of appreciable asymmetry of yield between the two halves of any one udder is much re duced as compared with goats receiving oestrogen alone Both these observations are suggestive of histological normality, an

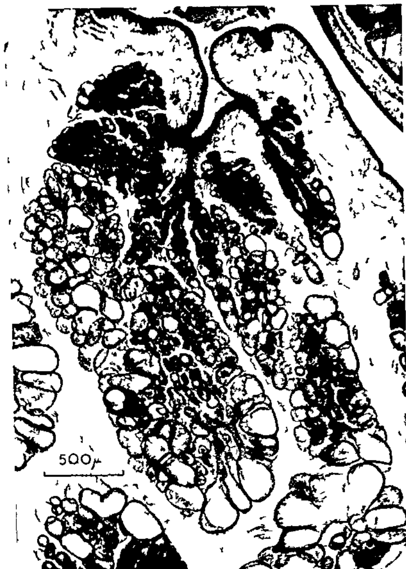
PLATE 1B




Section of an udder half from an ovariectomized virgin goat which had received 1·0 mg hexoestrol daily for 96 days (*photograph provided by Mr A. C. Richardson*)

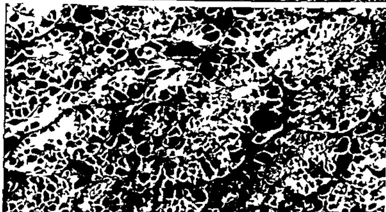
All workers in the field would agree that the anterior pituitary must be regarded as the mainspring of the hormonal mechanism governing the growth of the mammae in the normal female if only because it controls the secretion of those ovarian steroids long recognized as the essential instigators of mammary development But in addition to effects mediated by the ovary we must consider the possibility that the anterior pituitary can indirectly influence mammary development through the other target glands the adrenal cortex and thyroid

Studies of the effects of adrenalectomy on the structure of the mammary gland have given rather conflicting and by no means dramatic results and so have not contributed much information on the role of the adrenals in mammary growth Thus Chamorro (1946) could see no effects in the rat though we ourselves (Cowie and Folley 1947) like Trentin and Turner (1947) have observed regressive changes Other workers on the other hand have reported increased duct growth and budding after adrenalectomy (Reeder and Leonard, 1944, Johnston and Smithcors, 1948) As regards the mammogenic effects of steroids which have been isolated from the adrenal cortex, there is evidence from more than one laboratory (Van Heuverswyn, Folley and Gardner, 1939 Speert 1940) that deoxycorticosterone is active in this respect though contrary observations have been reported, notably by Chamorro (1945) However, it is doubtful whether deoxycorticosterone is secreted in significant amounts by the adrenal cortex under physiological conditions As regards the 11 oxygenated corticoids Flux (1954*b*) has recently shown in our laboratory that these steroids are not only devoid of mammogenic activity in the ovariectomized virgin mouse but that they also inhibit the growth promoting effect of exogenous oestrogen on the mammary ducts Cortisone also antagonizes the mammogenic effect of endogenous oestrogen since it inhibits mammary duct growth in intact female mice On the other hand 11 deoxycorticosterone acts synergistically with oestrogen in promoting mammary duct growth in the spayed mouse Plate I 12 illustrates these results, it shows at the same magnification whole mounts of comparable mammary glands from spayed mice which received the treatments in




An immature lobule from a gland developed in response to oestrogen and progesterone in an ovariectomized virgin goat note the peripheral distribution of enlarged alveoli (*from Cowie Folley Malpress and Richardson* 1952)







Sections from the udders of goats at the same magnification —

*Top Section* from the udder of a normal goat in lactation

*Middle Section* from an udder developed with hexoestrol and progesterone

*Bottom Section* from an udder developed with hexoestrol alone

*(from unpublished work of Mr K C Richardson to whom I am indebted for the photographs)*

dicated The two upper pictures show how cortisone markedly diminishes the mammogenic effect of administered oestrogen *while the two lower ones show* that deoxycorticosterone has the opposite effect However, there is reason to believe that the adrenal cortex can produce other steroids including oestro gens, progesterone and androgens which are known to possess mammogenic properties and it is worth noting in this connection that Nelson (1941) found that ACTH caused mammary growth in the spayed but not the spayed adrenalectomized rat

Nevertheless it seems doubtful whether the adrenals play a significant role in normal mammary development, since not only could we ourselves find no marked inhibition of mammary *growth in the rat as a result of adrenalectomy, but we could* detect no significant reduction in the mammogenic effect of an unfractionated ox anterior pituitary extract in adrenal ectomized rats (Cowie and Folley 1947) This conclusion has found a supporter in Jacobsohn (1949) on the basis of inter esting experiments on rats joined in parabiosis

Similarly as regards the thyroid there is no evidence that its secretions are essential for mammary growth though there are many findings somewhat complicated and even at first sight rather conflicting which suggest that the thyroid hor *mone may regulate the respective growth rates of both ducts* and alveoli in response to the appropriate stimuli Recent studies by various workers on the rat and mouse lead to the general conclusion that in the former hypothyroidism in duced by thyroidectomy or by administration of anti thyroid drugs leads to enhanced development of the alveoli and often but not always of the duct system as well, in response to oestro gen and progesterone either administered experimentally or endogenously produced while the administration of thyroxine has the opposite effect In the mouse on the other hand hypo thyroidism *seems to inhibit mammary development while* mild hyperthyroidism stimulates it As Trentin Hurst and Turner (1948) suggest the explanation may perhaps be sought in the relative thyroid secretion rates in the two forms, that in the normal rat probably being somewhat above the optimum for mammary growth while in the mouse the opposite may be assumed to be the case This hypothesis provides an intelli gible explanation of many apparent discrepancies among the




Abnormal folding of the alveolar epithelium found mainly in goat udders developed by high dosages of oestrogen without progesterone (*from Cowie Folley Malpress and Richardson* 1952)

mammary development in hypophysectomized animals It was from the results of these and other experiments that Turner s mammogen theory was developed To enlarge on the earl ier evidence for and against this still controversial theory is beyond the scope of this book A full discussion is given in the review by Folley (1952) It must suffice to point out now that in the light of continued experiment the theory has undergone important modifications at the hands of its originators They no longer believe that the anterior pituitary secretes two mammogens responsible respectively for duct and alveolar development the first of which unlike all other anterior pituitary hormones is a lipoid soluble substance Trentin and Turner (1948) now feel that both duct and alveolar growth can be ascribed to the action of a single pituitary factor or complex of factors associated with the protein fraction and they admit that the existence of a specific mammogenic hormone distinct from the six well known pituitary hormones cannot be regarded as conclusively established at the present time It may reasonably be said that the mammogen theory would carry more conviction if a specific mammogen could be separated from the pituitary in a state of reasonable chem ical and biological purity In any event as pituitary hor mones become available in an ever increasing state of purity our progress towards a deeper understanding of the role of the pituitary in mammary development must inevitably advance

Some progress towards this goal may be seen in experiments recently reported by Lyons and by Nelson which argue rather strongly against the need to postulate the existence of specific anterior pituitary mammogens Lyons (1951) successfully sought hormone combinations which would sustain lobule alveolar growth in virgin female rats subjected to a variety of operative procedures and found *inter alia* that lobule alveolar growth could be evoked in hypophysectomized ovariectomized rats by administration of oestrone progesterone and purified prolactin though the alveoli grew more extensively if a cruder prolactin containing ACTH and somatotrophin was used Plate I 13 shows part of the mammary gland of a rat possess ing neither ovaries nor pituitary and which was given oestrone, progesterone and prolactin This hormone combination pro duced quite extensive alveolar development but the complete

experimental results in this field and fits in with the concept of a regulatory role for the pituitary thyroid mechanism in mam mary growth

Our own experiments (Cowie and Folley 1947) provide evidence that anterior pituitary extracts can exert mammo genic effects that are mediated by neither ovaries nor adrenals since our unfractionated extract evoked mammary development in gonadectomized adrenalectomized rats, that is in animals which provided accessory cortical tissue was absent, possessed no known major source of steroids (steroprived rats) If then, these experiments can be accepted as decisive, the possibility that the pituitary does no more than play a so called permissive or sensitizing role or else a synergistic role in relation to the mammogenic steroids is excluded and we are left with a direct mammogenic effect as suggested for prolactin by the aforementioned intraduct injection experiments of Lyons (1942) If prolactin is indeed a direct mammogenic as well as lactogenic hormone it should be pointed out that neither our experiments nor those of Lyons exclude the possi bility of other pituitary hormones being also needed in a ' permissive or synergistic role, since in both cases animals possessing pituitaries were used In addition, the possibility that the pituitary growth hormone somatotrophin, is concern ed in mammary growth was suggested by the experiments of Nathanson, Shaw and Franseen (1939) and of Reece and Leonard (1941) and further evidence that this is so has re cently come to hand Lyons Li and Johnson (1952) have shown that somatotrophin augments the mammogenic action of the hormone triad oestrogen progesterone and prolactin in the hypophysectomized female rat (see also Lyons Li, Cole and Johnson 1953) The same group (Lyons, Johnson Cole and Li, 1955) have also shown that in the young hypophysec tomized male rat somatotrophin along with oestrogen and progesterone stimulate some degree of mammary duct growth even without prolactin

The question whether the anterior pituitary secretes specific mammogenic hormones distinct from its six well authenticated protein hormones was, as is well known raised by C W Tur ner and his school many years ago, principally because they found that ovarian steroids were ineffective in promoting



(a)



(b)





(c) 1 c m (d)

Second thoracic mammary glands from ovariectomized mice treated from the 21st day of age until the 42nd day photographs at the same magnification (*from Flux* 1953 1954)

| | | | |
|---|---|---|---|
| (*a*) | Oestrone 0 02 μg per day | (*c*) | Oestrone 0 01 μg per day |
| (*b*) | 0 02 μg + cortisone acetate 50 μg per day | (*d*) | 0 01 μg + deoxycorticosterone acetate 0 72 mg per day |

alveolar development typical of late pregnancy seemed to require somatotrophin in addition (Lyons *et al* 1952, 1953, 1955) The prolactin preparation could be replaced by one 12 day rat placenta daily, which suggests that in the later stages of pregnancy, at least in the rat, the placenta can assume the role of the pituitary in secreting prolactin or a substance with the same biological properties (Ray, Averill, Lyons and Johnson, 1955) The experiments of Nelson (1951) were main ly concerned with the hormonal maintenance of oestrogen induced pseudo pregnancy with its accompanying mammary development in virgin female rats variously treated and his results also permit of the conclusion that besides oestrogen and progesterone, only well recognised pituitary hormones, prin cipally prolactin but probably also ACTH and somatotro phin are required for complete lobule alveolar growth in the rat The design of both studies was such as to emphasize the probable dual role of prolactin in normal mammary growth the luteotrophic effect by which progesterone secretion is maintained and a direct 'mammogenic' action They also show that oestrogen and progesterone participate in mammary growth more directly than as mere pituitary excitants

Since many recent studies point to the hypothalamic con trol of pituitary function the possibility that neural and even psychological influences may exert an overriding influence on mammary development in the normal animal cannot be over looked This concept, formerly believed to be contra indicated once for all by mammary transplantation experiments such as those of Stricker (1929) which showed that the gland will grow in the absence of direct nervous connections must now be reconsidered in the light of the possibility that the mechan ism controlling mammary growth must be regarded as neuro endocrine in nature the endocrine phase comprising a com plicated and delicately balanced dynamic equilibrium centr ing round the anterior pituitary













Second thoracic mammary glands from ovariectomized mice treated from the 21st day of age until the 42nd day photographs at the same magnification (*from Flux* 1953 1954)

(*a*) Oestrone 0 02 $\mu$g per day
(*b*) 0 02 $\mu$g + cortisone acetate 50 $\mu$g per day
(*c*) Oestrone 0 01 $\mu$g per day
(*d*) 0 01 $\mu$g + deoxycorticosterone acetate 0 71 mg per day

PLATE I 13



Whole mount of a mammary gland from an hypophysectomized ovariectomized rat treated with oestrogen progesterone and a prolactin preparation (*from Lyons* 1951)

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## CHAPTER II

# THE INITIATION OF MILK SECRETION (LACTOGENESIS)

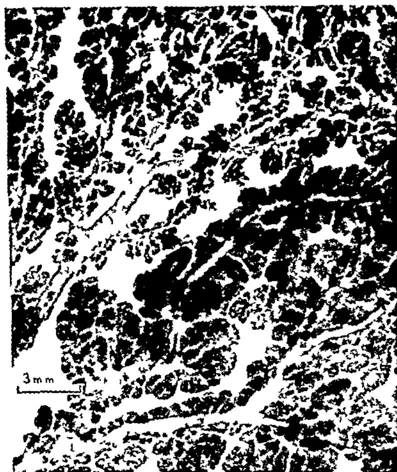
### THE BIOCHEMISTRY OF LACTOGENESIS

This chapter is concerned with the initiation of milk secr etion a process known as lactogenesis Experiments involving the removal of the pituitary gland performed many years ago showed that the integrity of the hypophysis is necessary for the initiation of milk secretion (for review see Folley 1952) Other experiments involving the administra *tion of pituitary extracts were even more illuminating for they* proved that the hypophysis provides a positive lactogenic stimulus The pioneer work in this latter field was that of Stricker and Grueter (1928) carried out in Bouin s laboratory at Strasbourg and this was soon followed by the general realization that anterior pituitary lactogenic extracts contain a specific hormone capable of initiating lactation in functionally competent mammary tissue This hormone is nowadays generally called prolactin but the alternative names, mammotrophin or luteotrophin are sometimes used An interesting first hand account by Stricker (1951) of what many regard as essentially the discovery of prolactin was recently given to a conference on the physiology of milk secretion appropriately held in Strasbourg

The prompt and perspicacious identification by Riddle Bates and Dykshorn (1933) of this presumed lactogenic hormone with a principle present in anterior-pituitary extracts, which induces enlargement and secretion of the pigeon crop gland provided the specific and convenient method of bioassay which is essential for rapid progress in the chemical purification of any new hormone In the case of prolactin this went forward to such good effect that it was the first pituitary protein hormone to be isolated as a pure or nearly pure protein

at least as judged by the criteria available at the present time In view of this it is not surprising that considerable progress has been made by now in elucidating the chemistry of prolactin

The ingenious and elegant experiments of Lyons (1942), later repeated by *Meites and Turner (1948), and in extensive* work in our laboratory by T R Bradley (unpublished) in which localized lactation was initiated in sectors of the rabbit mammary gland by the injection of small amounts of prolactin into the appropriate teat canal neighbouring untreated sectors remaining quite unaffected suggest that prolactin exerts a direct action on the mammary epithelium Plate II 1, taken from Lyons (1942) shows a portion of a whole mount of a mammary gland of one of his rabbits into one teat canal of which prolactin was injected The secretory hypertrophy of the alveoli of the treated sector, shown at the bottom right, contrasts with the undistended alveoli visible in the upper left of the picture However, since the rabbits used by Lyons were not hypophysectomized the possibility that other pituitary hormones of endogenous origin participated in the response was not excluded That other anterior pituitary hormones, in addition to prolactin are indeed concerned in lactogenesis is suggested by experiments carried out many years ago in the laboratories of Nelson Gaunt and Turner which showed that while systemic injections of unfractionated anterior pituitary extracts readily initiate milk secretion in hypophysectomized animals possessing the necessary mammary development, treatment with partially purified prolactin does not do this (Gomez and Turner, 1936, Nelson and Gaunt, 1936) Thus by the criterion customarily applied to the specificity of responses to anterior pituitary hormones namely that these should be limited to responses elicited in hypophysectomized animals, lactogenesis cannot be considered to be a specific response to prolactin alone Successful initiation of lactation achieved by the same groups of experimenters in hypophysectomized animals by administration of partially purified prolactin together with ACTH, adrenal cortex extracts or cortisone (e g Nelson, Gaunt and Schweizer 1943) shows that the pituitary adrenal mechanism is also involved in lactogenesis In view of this it is evident that until it is possible to




Lactogenic effect of injection of prolactin into a teat canal of a rabbit (*from Lyons* 1942)

work with undeniably pure pituitary hormones we cannot be certain whether other hypophysial functions may not be involved as well. The observations of Meites, Trentin and Turner (1942), who reported that adrenalectomy did not entirely prevent the onset of lactation in the rat at parturition, support this concept rather than otherwise since these workers admitted that the secretory activity in the absence of the adrenals was very feeble.

In view of what I have said it is perhaps more logical, as suggested by Folley and Young (1941), to regard lactogenesis as a response to the co-operative action of more than one anterior pituitary hormone rather than as a response to a single specific lactogenic hormone. In other words we should think in terms of a pituitary lactogenic hormone complex. It cannot be denied, however, that prolactin is the limiting factor in most experimental situations involving the animal possessing a pituitary gland though, as the experiments of Reece (1939) and others show, the experimental initiation of lactation in the pseudo-pregnant rat is an example of a case in which the intervention of the pituitary-adrenal mechanism in experimental lactogenesis is manifest. The pseudo-pregnant rat is notoriously refractory to the lactogenic action of prolactin preparations. Reece (1939) however, found it possible to initiate lactation in pseudo-pregnant rats by administration of partially purified prolactin together with adrenal cortex extract. More recently additional light has been thrown on the hormonal factors necessary for lactogenesis in the rat for Lyons, Li, Cole and Johnson (1953) have reported the experimental initiation of lactation in hypophysectomized ovariectomized virgin rats, possessing suitably developed mammary glands by treatment with prolactin, somatotrophin and ACTH or cortisone (see also Lyons, Johnson, Cole and Li (1955) for hypophysectomized male rats).

Further progress in gaining insight into the mechanism of lactogenesis must come from the exploration of biochemical pathways and the groundwork for advance along these lines is now being laid. Though abundant lactation does not set in until parturition or shortly thereafter, there is ample cytological and biochemical evidence of secretory changes in the mammary gland during the second half of pregnancy. Thus

the characteristic milk protein, casein, was detected by Cutler and Lewis (1933) using immunological methods, in the bovine udder fairly early in pregnancy Moreover, the gland at this stage can synthesize fatty acids from small molecules as shown by the isolation by Popjak and Beeckmans (1950) of labelled fatty acids from the mammae of pregnant rabbits given heavy water or $^{14}C$ acetate The late Dr T H French and I had the opportunity of demonstrating, in collaboration with Dr G Popjak, that these radioactive fatty acids contain

TABLE II 1

*Specific activities of the glyceride fatty acids from the mammae and livers of rabbits killed at the 28th day of pregnancy after treatment with $CH_3{}^{14}COONa$*

(from Popjak, Folley and French 1949)

| Fatty acid fraction | Specific activity of fatty acids $\mu c \times 10^{-4}$/mg C | | |
|---|---|---|---|
| | Rabbit no | | |
| | 10 | 13 | 15 |
| Volatile water soluble | 1 98 | 0 73 | 18 45 |
| Volatile water insoluble | 1 48 | 0 72 | 13 60 |
| Non volatile | 0 12 | 0 07 | 0 99 |
| Liver fatty acids | 0 146 | 0 183 | 0 58 |

ed the characteristic volatile short chain acids found only in milk fat and that, as shown in Table II 1, they were more radioactive than the non volatile long chain acids (Popják, Folley and French 1949) There is thus no doubt that the mammae of these pregnant rabbits were capable of synthesizing milk fat In accord with this, we have since shown in our laboratory (Table II 3) that mammary gland slices from pregnant rats will undoubtedly incorporate some $^{14}C$ into fatty acids if incubated with unlabelled glucose + [*carboxy* $^{14}C$] acetate even though the activity of the tissue in this respect may not be very great (Balmain Folley and Glascock, 1952) Another finding which can be cited here is the striking increase in the alkaline phosphatase content of the rat mammary gland dur

ing the second half of pregnancy, which is illustrated in Figure II 2 drawn from the results of Folley and Greenbaum (1947) This change however, is less easy to relate to any specific synthetic activity of the gland

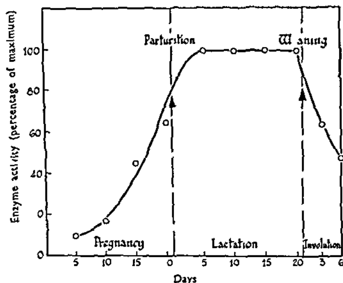



Fig II 2 Alkaline phosphatase content of the mammary gland of the rat *(from Folley and Greenbaum 1947)*

However despite this biochemical evidence of secretory activity in the mammary gland of the pregnant animal respiratory measurements on mammary gland slices from pregnant animals have given a picture of general metabolic quiescence contrasting with the much more active respiration hown *in vitro* during lactation This is illustrated by the results of Folley and French (1949*b*) summarized in Table II 2 Thus the endogenous respiration of mammary gland slices from rats at the end of pregnancy is low — $Q_{O_2}$ having a value of only 1 5 and it is not increased by the addition of glucose to the medium Moreover the respiratory quotient of the tissue at this stage is below unity values between 0 62 and 0 83

being found irrespective of whether or not glucose is present in the medium

Mammary tissue from lactating rats on the other hand, exhibits much greater respiratory activity *in vitro* The results in the Table show that by the first day *post partum* the tissue has acquired the power of utilizing glucose, in the presence of which substrate the respiratory quotient has risen to unity

TABLE II 2

*Respiratory metabolism of slices of rat mammary gland during pregnancy lactation and involution*

(from Folley and French, 1949*b*)

| Stage | Days | $-Q_{O_2}$ | | R Q | |
|---|---|---|---|---|---|
| | | Glucose (0 3 %) | | Glucose (0 3 %) | |
| | | + | — | + | — |
| Pregnancy | 20 | 1 3±0 1 | 1 5±0 05 | 0 83±0 01 | 0 62±0 03 |
| Lactation | 1 | 4 4±0 3 | 4 0±0 3 | 1 00±0 05 | 0 73±0 01 |
| | 8 | 7 1±0 6 | 4 5±0 5 | 1 62±0 03 | 0 76±0 01 |
| | 15 | 10 3±0 4 | 5 2±0 4 | 1 60±0 06 | 0 78±0 02 |
| | 22 | 9 6±0 3 | 6 3±0 2 | 1 53±0 03 | 0 74±0 02 |
| Involution | 2 | 5 3±0 9 | 5 0 | 0 76±0 03 | 0 64 |

By the 8th day $-Q_{O_2}$ in the presence of glucose has risen to 7 1 and the respiratory quotient is 1 62 at about which value it remains throughout lactation Respiratory quotients greater than unity in the presence of suitable substrates were also found for lactating mammary gland slices from the mouse, guinea pig rabbit cow goat and sheep (Folley and French 1949*a*, 1950) This is in general agreement with results previously obtained in arterio venous studies on the udder of the lactating cow by Petersen and Shaw (1942) and on that of the lactating goat by Graham, Houchin Peterson and Turner (1938) and later for the perfused, isolated cow udder by Peeters and Massart (1952)

The real changes in $-Q_{O_2}$, which is generally expressed as microlitres of oxygen consumed per milligramme final dry

weight per hour, are probably not so great as indicated by the results just discussed since mammary tissue from rats at the end of pregnancy contains much more metabolically inert dry matter, than mammary tissue from lactating rats The respiratory quotients are not subject to this error, however since the dry weight cancels out in the calculation In an attempt to overcome the difficulty of finding a suitable reference standard for oxygen consumption, we attempted to calculate the total respiration of the six abdominal mammae of the rat during pregnancy and lactation respectively and our values support the conclusion we drew from $Q_{O_2}$ measurements namely that the onset of secretory activity at parturition is indeed associated with a real increase in the rate of respiration of the tissue (Folley and French 1949*b*) Further support comes from the fact that we also found that the ratio of respiration to glycolysis ($-Q_{O_2}/Q_G^N$) is greater for mammary slices from lactating than from pregnant rats (Folley and French, 1949*b*) Also in harmony with this conclusion are the marked increases in the concentration of oxidative enzyme systems, succinoxidase and cytochrome oxidase about the time of parturition which have been observed by Moore and Nelson (1952) in studies of homogenates of rabbit mammary gland

Another quite striking way in which these changes in the respiratory metabolism of rat mammary tissue at the time of initiation of lactation can be illustrated is by manometric measurements of the time course of the total gas exchange which we call the composite respiration curve of mammary gland slices incubated in Krebs saline bicarbonate in equilibrium with 95 % oxygen and 5 % carbon dioxide and containing suitable substrates Figure II 3 shows some composite respiration curves for rat mammary gland slices obtained in our laboratory (Balmain and Folley 1952) It will be seen that the metabolism of mammary slices from 20 day pregnant rats is such as to give a slow fall in pressure under these conditions probably in the main because the respiratory quotient is less than unity and the slices therefore use oxygen at a greater rate than they evolve carbon dioxide Mammary slices from lactating rats, for which the respiratory quotient is greater than unity on the other hand give a steady increase in total pressure mainly because the slices give off carbon dioxide faster than they use up oxygen

Some years ago we interpreted the high respiratory quotient of lactating mammary tissue *in vitro*, in the presence of suitable substrates as evidence that this tissue possesses the power of effecting net fatty acid synthesis from small molecules (Folley and French, 1949*a* *b*, 1950) a conclusion which we later confirmed by isotopic assays on fatty acids isolated from mammary

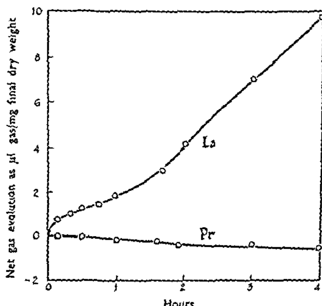



Fig II 3 Total gas exchange of mammary gland slices from rats killed on the 14th day of lactation (La) and 20th day of pregnancy (*Pr*) respectively The ordinate shows net gas evolution as µl gas/mg final dry weight uptake being calculated as $O_2$ and output as $CO_2$ (*from Balmain and Folley 1952*)

slices incubated with suitably labelled substrates Experiments involving the injection of labelled substrates into the lactating goat, cow and rabbit which are discussed in Chapter V have led to a similar conclusion We may therefore conclude that the changes in the respiratory metabolism of mammary tissue which begin at or shortly after parturition must be indicative among other things of a rapid acceleration of a lipogenic process already capable of proceeding at a relatively slow rate

In support of this conclusion we have shown that, even allowing for the probable dilution effect of the greater fat content of mammary tissue at the end of pregnancy, mammary gland slices from rats killed at days 1–4 *post partum* incorporate much more $^{14}C$ into the fatty acids, when incubated with glucose + [*carboxy* $^{14}C$] acetate than slices taken at the end of pregnancy Table II 3 shows results which illustrate this (Balmain, Folley and Glascock 1952) The Table shows that the specific activities of mixed fatty acids isolated from mammary gland slices

TABLE II 3

*Specific activities of mixed fatty acids of slices of mammary gland from rats killed at the end of pregnancy or in early lactation The substrates were* [carboxy $^{14}C$] *acetate + glucose*

(from Balmain, Folley and Glascock 1952)

| Experiment no | Stage | Period of incubation at 37 hr | $^{14}C$ in fatty acids counts/min/mg *C* | |
|---|---|---|---|---|
| | | | Incubated tissue | Tissue killed at zero time |
| 1 | pregnancy 20th day | 5–6 | 403 | 25 |
| 2 | , | 5–6 | 652 | 7 |
| 3 | | 5–6 | 407 | 7 |
| 4 | lactation 2nd day | 7 | 5 422 | 14 |
| 5 | lactation 4th day | 3 | 16 146 | 54 |

taken from rats killed shortly after parturition are many times higher than those found for tissue from rats killed at the end of pregnancy It is certain also that the rates of synthesis by the mammary tissue of characteristic milk constituents other than fat undergo similar sharp increases at parturition Thus Greenbaum and Greenwood (1954) have recently shown that the levels of glutamic dehydrogenase and of glutamic aspartic transaminase in the mammary gland of the rat dramatically increase immediately before parturition and remain at this enhanced level throughout lactation declining again only

after weaning These enzymes are believed to be concerned with protein synthesis in the cell and if this is so, then these changes are probably associated with the inception of a high rate of synthesis of milk proteins consequent upon the initia tion of lactation

Now, the biochemical changes observed in mammary tissue at the onset of lactation which I have just discussed, are un doubtedly evoked by the pituitary lactogenic hormone com plex which must come into full operation at parturition and they promise to afford a useful means of gaining further in sight into the hormonal mechanism of lactogenesis *in vitro* For instance the direct lactogenic action of prolactin upon the mammary epithelium so strikingly demonstrated in the rabbit by Lyons (1942) in the experiments described earlier in this chapter should be reproducible *in vitro* if the other hormonal influences operating in the normal environment of the mam

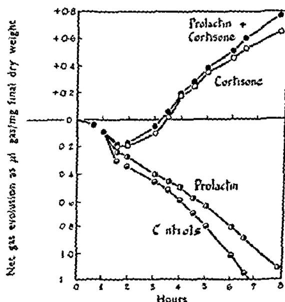



Fig II 4. Effects of cortisone (100 µg/ml ) and of prolactin (11 i u /ml ) on the respiration of mammary gland slices from a rat at the end of pregnancy *(from Balmain and Folley 1952)*

mary gland *in vivo* can also be reproduced In a preliminary attack upon this problem utilizing changes in the slope of the composite respiration curve as an indication of lactogenic effects, Miss Balmain and I were, however, unable to demonstrate any effects of prolactin, added *in vitro* on the respiratory metabolism of mammary slices from 20 day pregnant rats (Balmain and Folley 1952) A typical experiment is illustrated in Figure II 4 which shows that addition of prolactin to the medium had no effect on the slope of the composite respiration curve Rat mammary tissue, at this stage has thus proved to be insensitive to the action of prolactin *in vitro* by any criterion so far applied This of course may be because the correct conditions necessary to demonstrate its action have not yet been discovered On the other hand Figure II 4 also shows that cortisone, which on the basis of the *in vivo* work already mentioned might be expected to come into play as a result of the intervention of ACTH in lactogenesis when added to the incubation medium exerted definite effects on the composite respiration curve converting the falling pressure curve usually exhibited by mammary tissue from pregnant rats into the steadily rising one typical of secreting tissue This is the outcome one would expect if secretory changes had been initiated in the tissue by the hormone though it must be noted that the same result might be obtained if respiration were inhibited and glycolysis increased The two hormones together had no greater effect than cortisone alone so that these experiments provided no indication of the synergism between prolactin and the pituitary adrenal axis which had been inferred from the previous experiments of others on lactogenesis *in vivo* Nevertheless in so far as these findings appear to suggest a role for the adrenal cortex in the induction in rat mammary tissue of certain biochemical changes similar to those seen in tissue in a secretory state they are in accord with the aforementioned findings of Reece (1939) and others in the intact pseudo pregnant rat However experiments in our laboratory on mammary slices from pregnant rats which were incubated with labelled substrates have given results difficult to reconcile with the respiratory measurements just considered in that they suggest that cortisone in the concentrations used inhibits rather than promotes lipogenesis

from small molecules in mammary tissue from 20 day pregnant rats Hence, although this biochemical approach to the problem of lactogenesis is promising, the results so far obtained present certain difficulties in interpretation which it is hoped will disappear on further investigation

THE HORMONAL CONTROL OF LACTATION INDUCTION AT PARTURITION

We now turn to the consideration of some of the theories which have been put forward to explain the initiation of lactation at parturition and in particular its timing Although it is generally agreed that mammary growth proper being largely complete the secretory phase begins in the mammary gland during the second half of pregnancy, nevertheless lactation in the ordinarily understood sense of a copious flow of normal milk is not initiated until parturition or shortly there after The nature of the mechanism which ensures the correct timing of lactogenesis in this sense has intrigued investigators for many years and is not fully understood even yet Of the various theories which have been proposed space allows mention of only two and these can only be considered briefly

The theory proposed by Nelson (1936) for the guinea pig postulated oestrogen as the agent responsible for holding lactation in check during the latter half of pregnancy The high body levels of oestrogen known to obtain during pregnancy, were believed to inhibit the secretion of prolactin by the hypophysis and also to exercise a direct inhibitory influence on secretory changes in the mammary gland itself If this theory be extended, as proposed by Folley and Malpress (1947) to embrace the concept of two thresholds for opposing influences of oestrogen upon pituitary lactogenic function, a lower one for stimulation and a higher one for inhibition it will be found even yet to harmonize many experimental observations made on other forms besides the guinea pig on which most of Nelson s observations were based However in the light of recent results to be considered in more detail later it may be necessary to modify the theory still further to include an inhibitory role for progesterone in the capacity of a synergist with oestrogen

A somewhat more elaborate theory was proposed by Meites

and Turner (1942) on the basis of extensive investigations in Turner's laboratory of the prolactin content, as measured by a variant of the pigeon crop gland test, of the pituitary in various physiological and experimental states Briefly this theory rejects any inhibitory role for oestrogen in lactation, its authors rather see oestrogen as an indirect evocator of lacto genesis by virtue of its claimed ability even when circulating in high titres, to elicit the secretion of prolactin by the hypo physis while progesterone is regarded as the inhibiting agent operative during pregnancy with the power of nullifying or overriding the lactogenic action of oestrogen Lactogenesis is thus believed to result from a fall in the body level of pro gesterone relative to that of oestrogen which is thought to occur at parturition or shortly before Much evidence some aspects of which were later subjected to criticism (Folley 1952) was adduced in favour of this hypothesis in four papers published in 1942 and the theory has been restated with the support of additional experimental data and discussion in a more recent publication (Meites and Turner 1948) The scope of this book does not permit of discussion of this evi dence all that can be done is to state briefly the main finding of Meites and Turner This was that treatment with oestrogen even in unphysiologically high doses caused always an in crease never a decrease in the prolactin content of the pituitary in various laboratory mammals If, however, suffi cient progesterone was injected along with oestrogen no in crease in the pituitary prolactin content resulted Incidentally the above mentioned more recent publication of Meites and Turner (1948) contains no reference to a significant paper by Atkinson and Leathem (1946) providing evidence which partially answers criticisms made a few years ago (Folley 1952) of one of the vulnerable points of the Meites Turner theory namely, the lack of evidence for a fall in the body level of progesterone relative to that of oestrogen at parturition Using histological criteria Atkinson and Leathem could find little or no evidence of progesterone effects in the mouse on the day of parturition though signs of the presence of oestrogen were clear

However despite this, the author sees no reason to modify his previous opinion (Folley 1952) that while this ingenious

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or termination of lactation in women the decisive inhibitory factor has been held to be the removal of the suckling stimulus, the undoubted beneficial effects of the oestrogen which is often administered being ascribed to its ability to alleviate painful breast 'engorgement'

Some of those who hold that oestrogens have no inhibitory effect on lactation, at least under physiological conditions, have contended in support of their case that oestrogens are completely without effect on lactation in the absence of the

TABLE II 4

*Effect of progesterone on lactation in intact rats*

(from Folley 1942)

| Treatment | No of rats | % of young weaned (21 days) | Mean wt of young at weaning g |
|---|---|---|---|
| Sesame oil (control) | 4 | 97 | 42 9 |
| Progesterone 10 mg daily | 4 | 94 | 38 4 |
| Sesame oil (control) | 4 | 91 | 40 7 |
| Progesterone 15 mg daily | 3 | 92 | 40 7 |

All litters reduced to 8 at birth

ovary However careful consideration of the numerous papers which have been published on this subject shows that the true position is that while oestrogens will in some cases inhibit lac tation in the absence of the ovary provided high enough doses are given, nevertheless intact animals are undoubtedly much more sensitive to the lactation inhibiting effects of oestrogen than ovariectomized ones (e g Folley and Kon 1937) This suggests either that under physiological conditions proges terone itself might be the true lactation inhibitor or alter natively that it might act synergistically with oestrogen How ever many years ago the author found that progesterone by itself was quite ineffective as an inhibitor of lactation in spayed rats (Folley, 1942), as shown in Table II 4 from which it is evident that the young of rats receiving as much as 15 mg

theory can be regarded as perhaps the most successful attempt so far made to explain the mechanism of the initiation of lactation at parturition, the sum total of the available evidence in its favour is not even yet sufficiently unequivocal and convincing to justify the problem being regarded as finally solved The necessity for caution is underlined by results fairly recently reported by Meites and Sgouris (1953) which, taken at their face value are difficult to reconcile with the Meites Turner theory Mammary development was evoked in gonadectomized rabbits by administration of oestrone and progesterone in a favourable ratio Continuance of this regime prevented the initiation of lactation by prolactin in doses which were effective in the presence of either steroid alone According to the Meites Turner theory prolactin should have been just as effective in evoking lactation in the presence of oestrone and progesterone as in the presence of either hormone alone More recently still, in similar experiments, Meites and Sgouris (1954) have found that whether or not lactation occurs depends on the relative levels of prolactin on the one hand and the oestrogen progesterone combination on the other These findings appear to underline the necessity for some simplification of the original theory and Meites (1954) has recently outlined his latest views on the subject

This brings us back to the question of the nature of the hormonal influence which holds lactation in check during pregnancy Oestrogens have long been regarded as possessing the power of inhibiting lactation a concept upon which Nelson founded his theory of the mechanism of lactation initiation and which also motivated the widespread clinical use of oestrogens for preventing or inhibiting lactation in the puerperium However some workers have expressed doubts whether the ability to inhibit lactation is a direct and specific property of oestrogen, at least under physiological conditions Those holding this view have variously ascribed the apparent lactation inhibiting effects of oestrogens in small animals to anorexia or other secondary toxic effects on the mother, to interference with the milk ejection reflex (see chapter IV), or to untoward effects via the milk on the young whose growth rate is the only available measure of lactational performance in such laboratory animals as rats As regards the prevention

experiments on the artificial induction of lactation in farm animals on the topic under discussion In Chapter I reference was made to lactogenic effects elicited by oestrogens under suitable conditions These have been demonstrated most spectacularly in farm animals in which lactation has been initiated in udders grown by treatment with oestrogen *pari passu* with the continuance for a time of the oestrogen treatment Figure II 5 shows some lactation curves for cows and heifers brought into milk secretion by the subcutaneous implantation of pellets of stilboestrol (Folley and Malpress 1944) It will be seen that the best of these animals gave a total milk yield in a year of well over 700 gallons

Experiments carried out more recently in our laboratory on the spayed virgin goat (Cowie *et al* 1952) indicate that it is possible to select a daily dose of oestrogen for example 1 mg hexoestrol daily, which will cause mammary growth but at the same time tends to inhibit secretion in the sense that the unmilked udder does not become swollen with secretion in the latter stages of oestrogen treatment as often happens when a suitable lower dose is used The strong lactogenic effect of such a low dose of oestrogen is illustrated in the two lower pictures of Plate II 6 which shows photographs of the udders of two ovariectomized virgin goats in which udder growth was induced by the daily injection of 0·25 mg hexoestrol for 20 weeks These two udders are swollen with accumulated secretion The 1 mg daily dose of hexoestrol referred to above may be considered to have exceeded the upper of the two thresholds which Dr Malpress and I postulated in our double threshold theory while the 0·25 mg daily dose clearly came in the lactogenic zone between the two thresholds Moreover as the two much smaller udders in the upper pictures show the lactogenic effect of this latter dose of oestrogen was abolished completely by simultaneous administration of 100 mg progesterone daily These conclusions about the initiation of lactation in our goats which incidentally are in accord with findings in the rat reported by Selye (1940) depended on the gross appearance of the unmilked udder milking of which was not begun until the treatment ended except in the case of the two goats receiving the lactogenic dose of oestrogen whose udders became so tense

progesterone daily grow as fast as the young of control rats We are therefore left with the alternative possibility, predicted long ago by some of the earlier workers, that progesterone and oestrogen in combination act synergistically, the combination furnishing a far more potent inhibitor of lactation than oestrogen alone Fauvet (1941) seems to have been the first to provide experimental evidence for this concept when he showed that doses of oestrogen and progesterone, ineffective by themselves in combination effectively inhibited lactation in spayed female rats The ability of relatively small doses of oestrogen in combination with progesterone to inhibit lactation in the absence of the ovary, at least in the rat, has since been repeatedly confirmed Confirmation of this concept has also been obtained in the rabbit by Meites and Sgouris (1953, 1954) in the goat (Cowie, Folley, Malpress and Richardson, 1952) and in man by Romani and Recht (1948)

It will be of interest to consider now the bearing of some

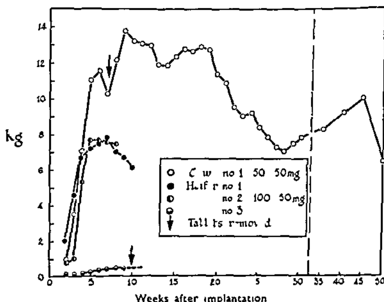



Fig II 5 Lactation curves for bovines implanted with tablets of diethylstilboestrol The ordinate shows mean daily milk yield *(from Folley and Malpress 1944)*

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as to necessitate milking before the end of the treatment period Results which in general confirm these conclusions have since been obtained in further experiments in goats in our laboratory (Benson, Cowie, Cox, Flux and Folley, 1955)

Reineke, Meites Cairy and Huffman (1952) have recently reported experiments on the artificial induction of lactation in cows in some of which truly spectacular milk yields were obtained Treatment with oestrogen and progesterone by pellet implantation was used to induce udder growth and then the animals were given a final short term treatment with oestrogen to initiate lactation in accordance with the Meites Turner theory of lactation initiation Table II 5, taken from the American workers' paper, shows the total yields of milk

TABLE II 5

*Induction of lactation in cattle by treatment with stilboestrol and progesterone*

(results taken from Reineke, Meites, Cairy and Huffman, 1952)

| Description of animal | Maximum daily milk yield lb | Total yield in 10 months | |
|---|---|---|---|
| | | milk lb | fat lb |
| Guernsey heifer J1 | 25 4 | 6681 | 323 |
| Guernsey heifer J2 | 24 7 | 6622 | 331 |
| Guernsey heifer J3 | 25 9 | 6532 | 369 |
| Holstein cow (Mabel) | 80 0 | 11330 | 420 |
| Holstein cow (Julia) | 45 0 | 7760 | 309 |

and fat obtained in ten months from six of their treated animals Their best animal, a sterile Friesian cow gave a peak daily yield of 80 lb and a total yield in ten months of over 1100 gallons The other five animals also gave good yields In view of these excellent results, a study has been undertaken in our laboratory of the effect of a final triggering treatment with oestrogen on the lactational performance of spayed goats in which udder growth was induced with oestrogen and progesterone (Benson *et al* 1955) Our results which are shown in




Photographs of the udders of four goats developed by injections of hexoestrol and progesterone The labels on each figure indicate the number of the goat and the daily dose of hexoestrol (HX) and progesterone (PG) in mg note the very large udders of goats 383 and 391 (*from Cowie Folley Malpress and Richardson* 1952)

Table II 6 indicate that though the extra oestrogen caused a slightly quicker initial rise in yield, the total yield over a long period was hardly affected The yield over 210 days can only be given for one udder half since the other half was removed for histological study as soon as the yield had reached its peak

TABLE II 6

*Induction of lactation in ovariectomized goats with hexoestrol and progesterone effect of final 'triggering' treatment with hexoestrol*

(results from Benson, Cowie, Cox Flux and Folley, 1955)

| Pair no | Milk yield | | | |
|---|---|---|---|---|
| | Whole udder over initial 28 days l | | Half udder over 210 days l | |
| | A | B | A | B |
| 1 | 25 5 | — | 134 | — |
| 2 | 31 3 | 40 9 | 208 | 194 |
| 3 | 23 1 | 21 1 | 134 | 136 |
| 4 | 15 9 | 27 3 | 114 | 156 |
| 5 | 25 4 | 30 6 | 134 | 128 |
| 6 | 21 8 | 18 4 | 122 | 129 |
| 7 | 17 6 | 22 9 | 92 | 111 |
| Mean | 22 9 | 26 9 | 134 | 142 |

A 0 5 mg hexoestrol + 70 mg progesterone daily for 150 days
B As above then 0 25 mg hexoestrol daily for 15 days

These experiments provide no evidence that a short term 'triggering' treatment with oestrogen following prolonged treatment with a combination of oestrogen and progesterone improves the subsequent lactational performance nor do our most recent experiments on virgin heifers the results of which are given in Table II 7 (Folley and Flux unpublished) These animals were given treatments very similar to that used by Meites and his colleagues but as can be seen their milk yields were not spectacular The comparative success of the infrequent injections of crystal suspensions is noteworthy

TABLE II 7

*Induction of lactation in heifers with oestrogen and progesterone*

(unpublished experiments of D S Flux and S J Folley 1953)

| Name of heifer | Treatment | 305 day yield | Peak daily yield |
|---|---|---|---|
| | | lb | lb |
| Winsome 31 | Oily injections | 5798 | 24 |
| Patty | Oily injections | 3987 | 21½ |
| Poise | Oily injections | 3349 | 18½ |
| Dinky | Tablet implants | 7578 | 39 |
| Joyce | Tablet implants | 3773 | 16 |
| Campion 27 | Tablet implants | 122½ | 3 |
| Nell 7 | Crystal injections | 7787 | 35½ |
| Marakatoria | Crystal injections | 3727 | 19½ |
| Primrose | Crystal injections | 4128 | 21½ |

*Injection of oily solutions*

0 5 mg hexoestrol + 70 mg progesterone in arachis oil daily for 120 days then 10 mg hexoestrol in oil daily for 16 days

*Tablet implants*

30 × 100 mg tablets progesterone + 1 × 100 mg tablet diethylstilboestrol for 120 days, + 15 × 100 mg tablets stilboestrol for the last 30 days

*Injection of crystal suspensions*

500 mg progesterone crystals at 30 day intervals over 120 days 100 mg oestradiol monobenzoate crystals on 1st day and 100 mg oestradiol monobenzoate in solution in oil on 90th day

In considering the impact of these recent findings with farm animals upon theories of the mechanism of lactation initiation it should be pointed out that some features of our own results with goats are in accord with the Meites Turner theory, while others are explicable on the basis of our double-threshold ' theory as are many previous results obtained on

cows as well as goats However, the recognition of the synergistic action of oestrogen and progesterone in inhibiting lactation as also the aforementioned experiments of Meites and Sgouris (1953, 1954) on the rabbit necessitate some revision of concepts I therefore venture to advance the following tentative theory, which combines various features of previous hypotheses and seems capable of harmonizing most of the known facts regarding the initiation of lactation The new theory embodies the following postulates (1) Measurements of the prolactin content of the hypophysis do not necessarily give any indication of the rate of prolactin release and are better disregarded as largely irrelevant to the issue (2) Low circulating levels of oestrogen activate the lactogenic function of the anterior pituitary while higher levels tend to inhibit lactation even in the absence of the ovary though it is uncertain whether this inhibition comes into play at the level of the pituitary or of its target organ the mammary gland or both (3) Lactogenic doses of oestrogen may be deprived of their lactogenic action by suitable doses of progesterone the combination then acting as a potent inhibitor of lactation This is the inhibiting influence which operates normally in pregnancy (4) The relative fall in the ratio of progesterone to oestrogen at parturition as demonstrated for the mouse by Atkinson and Leathem (1946) removes the inhibition which is replaced by the positive lactogenic effect of oestrogen acting unopposed

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## CHAPTER III

## THE MAINTENANCE OF MILK SECRETION — GALACTOPOIESIS

It is well known that the integrity of the pituitary is essential for the maintenance of milk secretion just as it is for its initiation This follows on the one hand from the immediate abolition of milk secretion which invariably follows hypophysectomy and on the other from the fact that, as we shall see later anterior pituitary extracts contain hormones capable of in

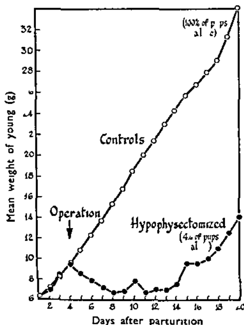



Fig III 1 Effect of hypophysectomy on lactation in the rat *(from unpublished experiments of A T Cowie)*

creasing the rate of milk secretion The dramatic effect of hypophysectomy upon milk secretion is illustrated in Figure III 1 which shows the results of an unpublished experiment on lactating rats carried out by Dr A T Cowie in our laboratory The Figure shows curves representing the mean growth rates of the litters of two groups of rats the members of one of which were hypophysectomized on the fourth day of lactation and thereafter given regular injections of oxytocin to cause milk ejection so that the litters could obtain milk if it was secreted It will be seen that growth of the sucklings ceased immediately after the mothers were operated upon and they soon began to die from lack of milk

It is however not yet certain whether the constellation of pituitary hormones responsible for the maintenance of estab lished milk secretion is identical with that concerned with its initiation If it is the same then active milk secretion could be regarded as a functional state of the morphologically com plete mammary gland which appears whenever the necessary pattern of pituitary hormones is present and unopposed by inhibiting influences a state which presumably would hold just so long as the pituitary continued to secrete the various components of the hormone complex in harmoniously balanced proportions If on the other hand the two pituitary constel lations are not identical then we must consider the possibility that prolactin exerts a trigger like action on the mammary gland which is only effective in the presence of other hypo physial influences perhaps regulating the supply of metabo lites whose continued intervention is necessary for the main tenance of secretion On this view it is conceivable that once lactation has been initiated prolactin is no longer essential for its continuance

In the light of present knowledge the first alternative seems preferable since it is in harmony with the theory proposed by Selye (1934) according to which the release of prolactin by the hypophysis is under neural control, the hormone being believed to be reflexly secreted by the anterior hypophysis in response to the suckling stimulus regular application of which is regarded as essential for the maintenance of milk secretion The facts upon which Selye based this theory were briefly as follows Suckling of some of the mammary glands of a lactating

rat was prevented either by surgical excision of the nipples or by covering them with collodion Provided other intact nipples belonging to the same animal were regularly suckled the unsuckled glands did not undergo the involution which in normal circumstances quickly follows weaning and at autopsy they were found to contain milk Moreover, if the escape of milk from the rat mammary gland was prevented by ligation of the main galactophores involution was retarded and a secretory condition maintained provided active suckling was continued Selye s theory, *the evidence for which cannot be considered in greater detail here**, thus lays emphasis on the loss of the suckling stimulus as the main cause of the regressive changes in mammary function and structure which follow weaning The fact that Williams (1945) later found it possible to mimic by prolactin administration the secretion maintaining effects of the suckling stimulus on glands which could not be emptied of milk or even suckled because of surgical intervention as just described not only supports Selye s theory but *also suggests that continued prolactin release is an important* factor in the maintenance as well as in the initiation of milk secretion It is of course likely that the secretion of other pituitary hormones believed to be involved in both processes may also be susceptible of control by the suckling stimulus Thus evidence that under some circumstances suckling may cause the release of one of them ACTH has been presented by Gregoire (1947)

As a result of simultaneous and independent suggestions of *Bergman and Turner (1940) and of Folley and Young (1940)* the term galactopoiesis has come into use to denote the experimental stimulation of established lactation The study of galactopoiesis is important since the hormonal mechanisms involved are probably closely related to those concerned in maintaining normal milk secretion and experimental analysis of the former will undoubtedly contribute to our understanding of fundamental aspects of the hormonal control of normal mammary function

PROLACTIN AND SOMATOTROPHIN

Unfractionated extracts of ox anterior lobe possess con

* For full discussion see Folley (1947)

siderable galactopoietic power in lactating c
single injections will evoke marked tempora
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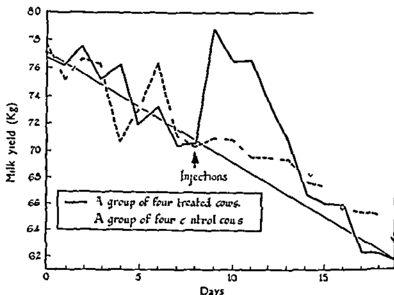



Fig III 2 Effect on the milk yield of cows of an injection of a saline extract of ox anterior pituitary *(from Folley and Young 1938)*

This Figure shows the milk yield increase exhibited by a group of four cows each of which received a single subcutaneous injection of 10 ml ox anterior pituitary extract At one time it was believed that the power of anterior pituitary extracts to increase the milk yield of lactating cows was mainly if not wholly due to the prolactin present in them However the results reported long ago by the Russian workers Azimov and Krouze (1937), implied that the galactopoietic effects of such extracts could not be quantitatively accounted for by the prolactin present and soon afterwards Young and the author studying a series of anterior pituitary extracts found very little relationship between their galactopoietic potencies in lactating cows and their prolactin contents as assayed by the pigeon crop test (Folley and Young 1938 1939 1940) In actual fact the galactopoietic potency of purified prolactin appears to be very small, at any rate as determined in single

tests in cows In the experiments of Cotes, Crichton Folley and Young (1949), cows in declining lactation, known to be responsive to unfractionated ox pituitary extracts gave no significant increases in milk yield in response to single injections of about 1000 i u purified prolactin (see Figure III 3) Though it may be objected that the dose used in these experiments was not a particularly high dose per kg, nevertheless we have found that amounts of unfractionated anterior pituitary extract containing far less prolactin regularly evoke considerable galactopoietic responses in similar cows Other

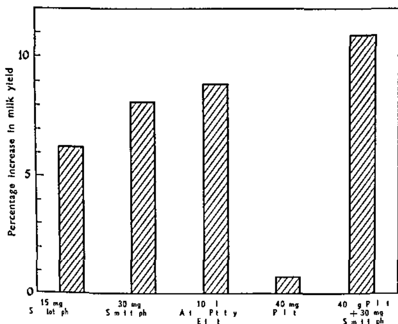



Fig III 3 Effect on milk yield in the cow of injections of hormones of the anterior pituitary *(this figure is constructed from the experimental results of Cotes Crichton Folley and Young 1949)*

work leading to the same conclusion is reviewed by Folley (1955) As a result of our earlier investigations Young and I concluded that the galactopoietic activity of unfractionated ox pituitary extracts must be due to the action of more than one hormone and so we arrived at the concept of a pituitary galactopoietic hormone complex (Folley and Young 1941)

As progressively more highly purified preparations of anterior pituitary hormones have become available over the years, Young and I with our collaborators, have attempted to determine the nature of the hormones, present in unfractionated ox anterior pituitary extracts, which are responsible for their galactopoietic activity In the earlier work with comparatively crude extracts galactopoietic activity was often found to be associated with diabetogenic activity When, therefore Cotes Reid and Young (1949) succeeded in showing that purified pituitary growth hormone, somatotrophin, possessed diabetogenic activity, it was clearly important to find out if pure growth hormone was galactopoietic in lactating cows Figure III 3 shows some results of experiments in which single injections of purified somatotrophin were administered to lactating cows These experiments were carried out by Cotes, Crichton Folley and Young (1949) on groups of cows at two experimental farms The responses are expressed in terms of the percentage increase in milk yield observed during the two days following the injection corrected for the change in yield of control cows receiving injections of saline The histogram shows that, in accord with expectation, single injections of 30 or 60 mg purified somatotrophin caused marked temporary increases in milk yield The magnitude of the response to these single injections of somatotrophin was such as to suggest that all or practically all of the galactopoietic activity exhibited by unfractionated ox anterior pituitary extract in single injection tests could be attributed to the growth hormone present Moreover there was no indication of a synergistic effect when growth hormone was administered together with prolactin or ACTH The probable fundamental significance of the galactopoietic activity of growth hormone is illustrated by the neat way in which its discovery, since confirmed in a number of laboratories (see Folley, 1955 for review), fulfils a prediction of Young (1947), who pointed out that galactopoiesis growth and diabetogenesis, involving as they all do the preservation of foodstuffs from oxidation, are phenomena which may be expected to owe allegiance to closely related hormonal regulating mechanisms

The finding that prolactin possesses relatively little galactopoietic activity in lactating cows, at any rate in short term

experiments, must not be taken as conflicting with the view expressed above, namely that its continued secretion is probably essential for the maintenance of lactation Though prolactin does not appear to be a limiting factor in the cow in the declining phase of lactation it might well be a limiting factor in certain circumstances in other species Despite some positive results, its value for the treatment of hypogalactia in women is far from certain but in this connection it should be noted that it is often not clear whether hypogalactia should be regarded as essentially a problem of lactogenesis or of galactopoiesis

In our laboratory we have recently attempted a biochemical approach to the problem of the role of prolactin in the maintenance of a secretory state in the mammary gland In the rat, the early stage of lactation days 1–5 is a period of biochemical lability in which, as shown in Chapter II the respiratory quotient of mammary tissue *in vitro* is increasing above unity and the slope of the composite respiration curve in bicarbonate buffer is changing from negative to positive We reasoned therefore that mammary tissue at this stage should be a favourable medium for demonstrating galactopoietic effects of prolactin *in vitro* Miss Balmain and I accordingly investigated in preliminary experiments the effect of prolactin added to the medium on the composite respiration curve of rat mammary gland slices in early lactation (Balmain and Folley, 1952) In our experiments equilibration with prolactin significantly increased the slope of the composite respiration curve resulting from the metabolism of mammary gland slices taken from rats autopsied during days 1–5 *post partum* Figure III 4 illustrates the results of one of these experiments It shows curves representing the overall pressure changes resulting from the metabolism of mammary gland slices taken from a rat killed on the fourth day of lactation incubated in Krebs saline bicarbonate, the substrate being a mixture of acetate and glucose It will be seen that the total pressure progressively increases and that addition of prolactin significantly increases the rate of pressure rise One is probably justified in regarding the effects observed in these experiments as essentially galactopoietic rather than lactogenic in nature but some recent experiments in our laboratory by Dr T R Bradley raise doubts whether they are due to the action of

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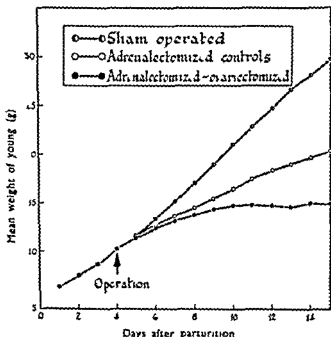



Fig III 6 Comparison between the effects of adrenalectomy and of adrenalectomy + ovariectomy on lactation in the rat *(results from Flux 1955)*

Replacement studies with adrenal corticoids in adrenalectomized lactating rats have given somewhat discordant results Though it was at first believed that the effect of the adrenals on lactation was mediated mainly through electrolyte and water metabolism Gaunt Eversole and Kendall (1942) later concluded that the critical factor for normal lactation was a sufficiency of 11-oxygenated steroids then believed to be mainly concerned with protein and carbohydrate metabolism They arrived at this conclusion because they could achieve complete lactation maintenance in rats adrenalectomized just after parturition with 11-oxygenated steroids but not with deoxycorticosterone In contrast Dr Cowie and the

suggested that single injections of ACTH-protein temporarily decreased rather than increased the milk yield of lactating cows The availability of very active low molecular preparations of ACTH has since enabled us to show quite conclusively that this is indeed the case Figure III 5 shows the results of a recent experiment with long acting ACTH carried out in our laboratory (Flux, Folley and Rowland, 1954) The curves shown in this Figure depict the mean daily milk yields of three groups of cows, two of which received ACTH while the third served as a control group The curves for the groups of cows receiving injections of 100 or 200 i u ACTH on the occasions indicated by the appropriate arrows show that either dose of the hormone caused a marked though temporary depression in milk yield

However although no galactopoietic responses to ACTH have been observed in cows studies of the effects of adrenalectomy on lactation in small animals show that the pituitary adrenal axis is a critical factor in lactation maintenance just as it is in lactogenesis This is attested by numerous experiments on small animals which have shown that adrenalectomy causes a marked though not complete inhibition of lactation Paired feeding experiments on the rat though presenting difficulties in interpretation because the adrenalectomized rat has less ability to support lactation by the catabolism of body tissues (as well as to expend energy in spontaneous activity) than pair fed controls indicated that part of this decline could be attributed to chronic anorexia resulting from loss of the adrenals leaving a definite though relatively small proportion to be ascribed to the loss of the adrenals *per se* (Cowie and Folley 1948) However recent experiments in our laboratory (Flux, 1955) show that an ovarian secretion probably progesterone, contributes significantly to the partial lactation maintenance observed after adrenalectomy for when the ovaries are removed as well as the adrenals lactation is much more severely inhibited than it is when only the adrenals are removed This is shown in Figure III 6 which shows the mean growth curves of the litters of three groups of rats comprising sham-operated controls, rats adrenalectomized on the fourth day of lactation and rats from which both ovaries and adrenals were removed on the fourth day It is obvious that lactation,

as judged by the growth curves of the young was most severely inhibited in the latter group

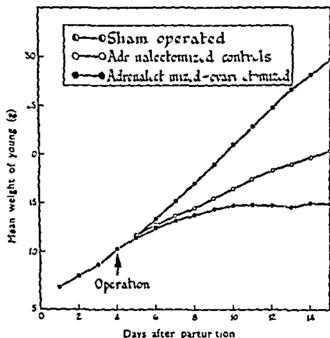



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author have never been able to achieve complete lactation replacement in our rats with 11-oxygenated steroids alone (Cowie and Folley, 1947, Cowie, 1952) In our earlier experiments, when scarcity of supplies limited the doses of 11 oxygenated steroids we were able to give, our most effective steroid was deoxycorticosterone, though even high doses of this substance did not completely restore lactation to normal Later, however, Cowie (1952) was able to show that larger doses of cortisone were as effective in lactation maintenance as deoxycorticosterone though as emphasized above the restoration was not complete

We have tried to discover the reason why the adrenalectomized rats of Gaunt, Eversole and Kendall (1942) lactate normally on cortisone therapy while ours do not The superiority of deoxycorticosterone in our earlier experiments was maintained when our rats were given a high protein diet with a view to providing conditions most favourable for the action of the glucocorticoids (Cowie and Folley, 1947) Cowie (1952) later found that considerable variations in the dietary intake of sodium also failed to affect the issue appreciably Thus, despite the fact that Nagareda and Gaunt (1948) found the lactation maintaining ability of deoxycorticosterone in their adrenalectomized rats to be sensitive to extreme variations in dietary sodium and potassium it seems hardly likely that differences in the dietary regimes obtaining in the respective laboratories could be large enough to account for these discrepancies Since, as shown in the Figure III 7 Cowie (1952) has now achieved virtually complete lactation maintenance in our rats by simultaneous administration of cortisone and deoxycorticosterone it seems possible that the explanation might lie in strain differences in the nature of the above mentioned contribution of the ovaries This Figure shows the mean growth curve of the litters of a group of rats adrenalectomized on the fourth day of lactation and thereafter given cortisone and deoxycorticosterone acetate by pellet implantation It will be seen that the litters of these rats grew as fast as those of sham operated control rats The discovery of the new adrenocorticoid, aldosterone, raised the question whether this substance was specially active in maintaining lactation in adrenalectomized rats Cowie and Tindal (1955) found

definite activity with as little as 50 $\mu$g daily but even more striking was their finding that 100 $\mu$g daily of 9α chloro hydrocortisone gave almost complete maintenance

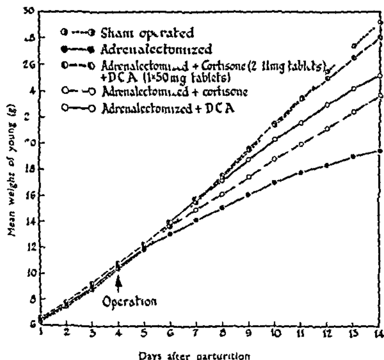



Fig III 7 Maintenance of lactation with adrenal corticoids in adrenalectomized rats *(from Cowie 1952)*

Biochemical studies are beginning to provide some information about the way in which the adrenal corticoids may be involved in the regulation of normal mammary function though such explorations are as yet only in their infancy Studies on the mammary gland arginase, an enzyme which in the liver is considered to be intimately involved in either the anabolic or catabolic phases of protein metabolism or perhaps both, at one time seemed promising but the latest results have given rise to a position less easy to evaluate Figure III 8 shows ome results of determinations of the arginase activity of rat

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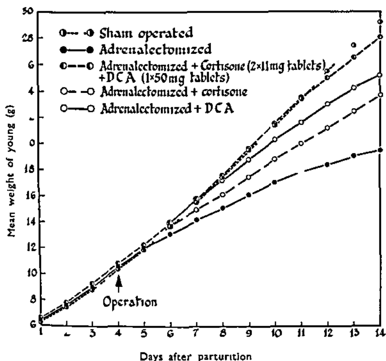



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mammary tissue carried out a few years ago in collaboration with Dr A L Greenbaum (Folley and Greenbaum, 1947) It will be seen that the arginase activity of the mammary tissue remains virtually constant at a relatively low level during pregnancy and for a few days *post partum* At about the fifth day *post partum* the enzyme content begins to rise rapidly and by the 20th day has attained a level about 5–6 times that at

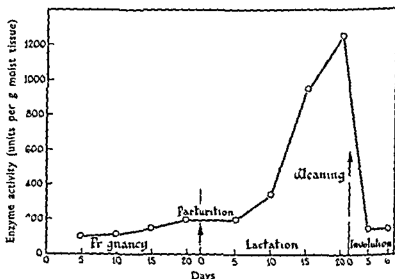



Fig III 8 Arginase content of the rat mammary gland during pregnancy and lactation *(from Folley and Greenbaum 1947)*

parturition decreasing sharply again after weaning In the rat in full lactation the arginase content of the mammary gland is quite notable this tissue being second only to the liver as a source of arginase in the body and having about one ninth of its activity Though the biological role of arginase in the mammary gland is not known with certainty, as we shall see, these results suggest a close relationship to the secretory process The same applies to the mammary gland of the mouse since the arginase content of the latter also increases as lactation proceeds and is comparable with that of the rat (unpublished results of Folley and Greenbaum, cited by

Folley, 1949) Adrenalectomy at the fourth day of lactation largely prevents the increase in the mammary gland arginase with advancing lactation since as Table III 1 shows the mammary arginase levels of adrenalectomized rats killed at the 17th day are much lower than those of pair fed controls

TABLE III 1

*Effect of adrenalectomy on the arginase content of the mammary gland of the lactating rat*

(from Folley and Greenbaum, 1948)

| | No of rats | Arginase content of mammary gland units/g milk free tissue |
|---|---|---|
| Sham operation (fed *ad lib*) | 8 | 162±9 |
| Sham operation (pair fed) | 9 | 147±10 |
| Adrenalectomized at day 4 of lactation (fed *ad lib*) | 9 | 20±2 3 |

(Folley and Greenbaum 1948) Lactation seems also to demand an increase in the liver arginase levels at least in the rat since as the curves in Figure III 9 show the liver arginase level increases by about 50 % immediately after parturition at which enhanced level it remains virtually constant through out lactation decreasing again only after weaning (Folley and Greenbaum 1947) Here also, as originally found by Fraenkel Conrat, Simpson and Evans (1943) for the livers of non lactating rats adrenalectomy at the fourth day decreases the liver arginase level to about one third of that of pair fed controls

The liver arginase is believed to be a component of a complicated enzyme system concerned with the deamination of amino acids so that it seems logical to interpret the increase

in the liver arginase level observed in the rat at about the time of parturition as reflecting an increase in the rate of hepatic gluconeogenesis This would probably result from an increased demand for deaminized residues which can be transported to

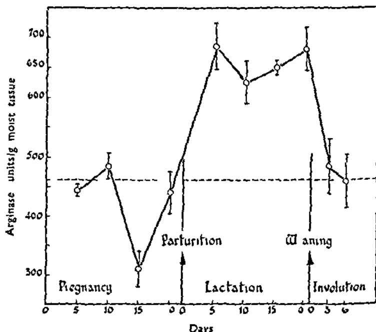



Fig III 9 Liver arginase levels in the rat during pregnancy and lactation *(from Folley and Greenbaum 1947)*

the mammary gland and there converted to lactose or milk fat or used for energy production This view involve the assumption that changes in the arginase level reflect changes in the rate of gluconeogenesis from protein Folley and Greenbaum (1947) likewise interpreted the changes in the arginase levels of the mammary gland of the rat during lactation and after weaning as evidence that the arginase of the mammary gland plays a similar role there This seemed all the more likely since Graham, Houchin and Turner (1937), on the basis of arterio venous studies, had earlier reported that the udder of the lactating goat produces urea It thus appeared reasonable to suppose that as the milk yield of the rat pro

gressively increases the liver fails to keep pace with the ever-increasing demands of the mammary glands for non nitroge nous milk precursors so that the mammary tissue is forced to realize to an increasing extent its potentialities (manifested by the presence of arginase) for effecting gluconeogenesis from protein On this view since the adrenal cortex through the action of the glucocorticoids is believed to promote gluconeo genesis the unfavourable effects of adrenalectomy upon lactation would be mediated through the resulting inhibition of gluconeogenesis from protein both in the liver and in mammary gland itself

However determinations of the arginase content of the lactating mammary gland in ruminants and other herbivo-rous animals have shown that the mammary tissue in these species contains very little enzyme as compared with the mammae of the rat and mouse This is evident from some unpublished results on the arginase levels of mammary tissue in various species obtained in collaboration with Dr Green baum (see Folley 1949) which are presented in Table III 2

TABLE III 2

*Arginase content of the mammary gland in various species during lactation*

(unpublished results of Folley and Greenbaum 1948 cited by Folley 1949)

| Animal | Stage of lactation (days) | No of animals | Units of arginase/g moist tissue | |
|---|---|---|---|---|
| | | | Absolute | Relative |
| Rat | 17 | 5 | 144 7 | 100 |
| Mouse | 5 | 5 | 51 0 | 35 |
| Mouse | 15 | 6 | 89 2 | 62 |
| Guinea pig | 5 | 5 | 7 8 | 5 |
| Guinea pig | 10 | 6 | 10 0 | 7 |
| Rabbit | 7 | 6 | 1 9 | 1 |
| Rabbit | 28 | 5 | 4 0 | 3 |
| Sheep | early | 3 | 3 9 | 3 |
| Goat | advanced | 6 | 3 2 | 2 |
| Cow | advanced | 10 | 2 0 | 1 |

In view of this finding it would seem that despite the contention of Graham and his collaborators that the lactating goat udder produces urea, it must be regarded as doubtful whether gluconeogenesis in the mammary gland plays any significant role in lactation in herbivorous forms In this connection it would be interesting to know how severe are the effects of adrenalectomy upon lactation in ruminants Moreover, as far as the rat itself is concerned we have found that the relative abilities of cortisone and deoxycorticosterone to maintain the tissue arginase levels in lactating rats after adrenalectomy are somewhat different from their relative lactation maintaining abilities in the same rats (Folley and Watson 1951) It therefore follows that even in the rat and mouse the only forms in which appreciable levels of arginase have so far been found in the mammary gland the biological role of arginase in this organ is still far from clear

Other biochemical studies which we have made suggest that the adrenal cortex may also be concerned in the regulation of a process for which the mammary gland exhibits outstanding capabilities, namely, lipogenesis If lactating rats are injected with [*carboxy*-$^{14}$C] acetate, the fatty acids isolated from the mammae at autopsy shortly thereafter are strongly radioactive, the specific activity of the short chain (volatile) fraction being greater than that of the long chain (non volatile) acids Unpublished studies carried out by Dr R F Glascock and Dr W G Duncombe in our laboratory have shown that after adrenalectomy the ratio of the specific activities of the volatile and non volatile fractions isolated from the mammary tissue of rats receiving radioactive acetate was higher than that of similar fractions isolated from the mammae of intact controls, suggesting that adrenalectomy had in some way disturbed the mechanism of lipogenesis from small molecules in the mammary gland Studies of the effect of adrenal corticoids in lipogenesis in mammary gland slices *in vitro* are in harmony with this supposition, but will not be further touched upon here since lipogenesis in the mammary gland is dealt with in detail in Chapter V

#### THE PITUITARY THYROID MECHANISM

We turn now to the role of the pituitary thyroid mechanism

in milk secretion Earlier work on the role of the thyroid gland in milk secretion led to the conclusion that while the thyroid hormone is not essential for mammary gland function nevertheless the pituitary thyroid axis may be regarded as an important regulator of the rate of milk secretion This conclu sion follows from the facts that on the one hand thyroidectomy reduces the milk yield but does not abolish secretion altogether, while on the other administration of thyroid hormone exerts a galactopoietic effect under suitable conditions The latter effect, first reported sixty years ago for the cow by Hertoghe (1896) has been confirmed and exhaustively investigated in recent years (see Blaxter 1952 for detailed review) It has also been recently demonstrated in rats by Desclin (1949) and women by Romani Plocq and Recht (1949) and by Roche Giraud Lelong, Liardet and Coignet (1950) Galactopoietic responses in cows were obtained by Folley and Young (1938 1939) by injections of pituitary extracts rich in thyrotrophin but virtually free from prolactin and this indication of the importance of the pituitary thyroid axis as a regulator of

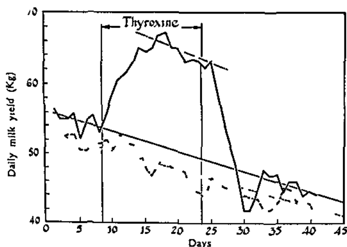



Fig III 10 Increase in milk yield of a group of four cows receiving DL-thyroxine sodium (10 mg/day) subcutaneously continuous line daily milk production of treated cows interrupted line daily milk production of control cows *(from Folley and White 1936)*

lactation is in harmony with the powerful galactopoieti action of the thyroid hormone which will now be considerec

The possibility of using thyroid active materials on a larg scale for increasing the milk yield of cows in declining lactatio has led to numerous experiments during the past decade o various aspects of the galactopoietic effect of thyroid hormone

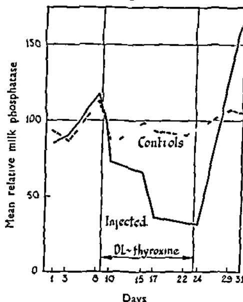



Fig III 11 Decrease in the alkaline phosphatase content of the milk of the cow after injections of DL thyroxine sodium (10 mg/day) The results are expressed as a percentage of the mean initial value Each curve represents the mean values for four cows *(from Folley and White 1936)*

The original work of Hertoghe was lost sight of until 1934 when the phenomenon was rediscovered by Graham (1934 *a, b*), who besides obtaining galactopoietic effects in cows by feeding dried thyroid gland showed that injection of synthetic thyroxine gave similar results Shortly afterwards we confirmed and extended Graham's work in our laboratory showing that daily injection of thyroxine not only caused a considerable temporary increase in milk yield but also increased the percent

ages of fat and non fatty solids in the milk (Folley and White 1936) Some of our results are shown in Figure III 10 in which the curves represent mean values for a group of cows receiving daily subcutaneous injections of 10 mg DL-thyroxine as compared with the values for a control group injected with saline We also showed that the concentration of alkaline phosphatase in the milk was dramatically reduced during the treatment period This is shown in Figure III 11 which illus trates the mean percentage drop in the milk alkaline phospha tase level This latter is a specific and sensitive response to the thyroid hormone which we later found very useful for assaying thyroid active preparations for galactopoietic potency in cows, its significance from the point of view of the mechanism of the interaction of the thyroid hormone and the mammary gland is unknown and would repay further study

Studies in many laboratories of the effect of thyroid active materials on milk composition have led to the conclusion that the only changes of note besides those already mentioned are a decrease in ascorbic acid content and as shown by Chanda and Owen (1951) an increase in the organically bound phosphate The experiments of Chanda McNaught and Owen (1952) and also those of Bailey Bartlett Folley Rowland and Thompson (1951) in our Institute have estab lished that during the period of treatment with thyroid hor mone the proportion of the vitamin $B_1$ of the milk present as co carboxylase that is in the phosphorylated form, increases concomitantly with the decrease in the alkaline phosphatase content Figure III 12 shows some of our results which illus trate this These results were obtained on a group of cows receiving L thyroxine by mouth The opposite changes in the alkaline phosphatase content and in the ratio of the phosphory lated to free aneurin in the milk are clearly shown One is tempted to conclude that the unphosphorylated aneurin of the milk arises by dephosphorylation of aneurin phosphate while the milk is in the udder However since aneurin phos phate is a pyrophosphate its dephosphorylation would presum ably require the successive action of a specific pyrophos phatase and of the alkaline phosphatase While the presence of a pyrophosphatase in cow s milk has been claimed this contention has not yet been satisfactorily proved Moreover

the alkaline phosphatase, the optimum pH of which is around pH 9–10, would probably not be very active at the pH of milk This attractive hypothesis thus requires further proof before it can be accepted

The earlier experiments on the galactopoietic effect of thyroid hormone mainly involved the feeding of iodocasein (see Blaxter, 1952 for review), which although readily made in

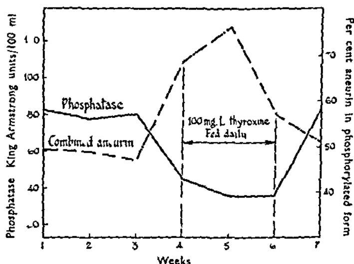



Fig III 12 The effect of feeding L-thyroxine on the alkaline phosphatase content of the milk of the cow and on the ratio of the phosphorylated to free aneurin *(from Bailey Bartlett Folley Rowland and Thompson unpublished results cited by Folley 1951)*

quantity and therefore cheap, possesses certain disadvantages in practice Thus, it must be standardized by biological methods often a troublesome and uncertain procedure, and some preparations possess a taste and smell which cause a proportion of cows to refuse it Moreover its administration entails a considerable intake of non thyroxine iodine which may cause untoward effects It had come into wide use mainly because synthetic thyroxine was until recently scarce and expensive and, moreover, believed to be relatively inactive by the oral route in cows A few years ago, however, Chalmers Dickson, Elks and Hems (1949) described a new and improved method

of synthesis of L-thyroxine which promises to make the hor mone available in quantity at a price which will compete with that of iodocasein This noteworthy advance prompted us to investigate the galactopoietic activity of L thyroxine when *given to lactating cows by the oral route and we found that its* potency by this mode of administration was much greater than had been supposed (Bailey Bartlett and Folley, 1949)

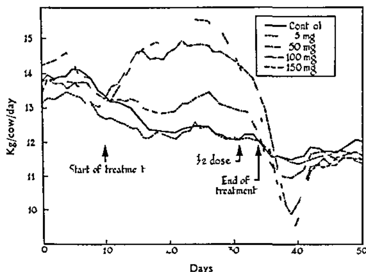



Fig III 13 Effect of L thyroxine given by mouth on the milk yield of groups of cows (dose levels indicated were fed daily) *(from Bailey Bartlett and Folley 1949)*

Our results are shown in Figure III 13 which shows the mean lactation curves for groups of cows receiving various daily doses of synthetic L-thyroxine by mouth It will be seen that a daily dose of 100 mg L thyroxine by mouth caused a conside rable temporary increase in milk yield, which incidentally was a little greater than the effect produced by feeding our stand ard dose of 20 g iodocasein daily Since thyroxine is odour less and virtually tasteless and since its purity can be checked by chemical techniques thus obviating troublesome bioassay procedures and since moreover, its use entails a relatively small iodine intake so that the risk of iodism is virtually

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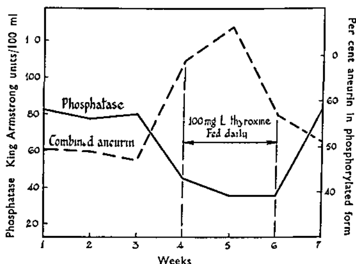



Fig III 12 The effect of feeding L-thyroxine on the alkaline phosphatase content of the milk of the cow and on the ratio of the phosphorylated to free aneurin *(from Bailey Bartlett Folley Rowland and Thompson unpublished results cited by Folley 1951)*

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inactivated in the rumen than thyroxine The first possibility was studied in an experiment in which the galactopoietic effects of equal doses, 5 mg daily, of the two substances given

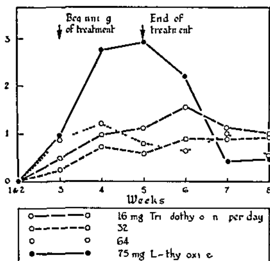



Fig III 14 Effect of feeding L triiodothyronine on the milk yield of groups of cows the curves represent changes in the milk yield in kg/cow/day *(from Bartlett Burt Folley and Rowland 1953)*

by subcutaneous injection were compared in lactating cows (Bartlett *et al* 1953) By the subcutaneous route 3 5 3 triiodo L thyronine proved to be somewhat more active than thyroxine by all criteria studied – the increase in milk yield and in the percentages of milk fat and non fatty solids the decrease in the milk alkaline phosphatase and the increase in the heart rate – though at the most its potency was no more than twice that of thyroxine To illustrate these results I have this time selected the effects on the milk phosphatase content which are shown in Figure III 15 The curves here represent the percentage changes in the milk phosphatase due to the treatment and you will note that 5 mg 3 5 3 triiodo L thyronine subcutaneously daily caused a slightly larger de

eliminated, L thyroxine is to be preferred to iodocasein for all purposes for which the latter has been or is intended to be used, in fact iodocasein can now be regarded as of academic interest only It is worth noting that reassuring results were obtained by Leech and Bailey (1953) in a large scale experiment in which the effects on the health and reproductive abilities of cows, of administration of L thyroxine in three successive lactations, were studied

Recently Roche, Lissitzky and Michel (1952) and Gross and Pitt Rivers (1953*a*) have independently isolated a biologically active material from the thyroid gland which has been identified as 3 5 3 triiodo L thyronine This substance which differs from thyroxine in chemical constitution only in that its molecule contains one less atom of iodine, has been reported to be 5–7 times more active than thyroxine in various biological tests in small animals and also in humans (Gross and Pitt Rivers 1953 *b*, Tomich and Woollett, 1953, Lerman, 1953) Since 3 5 3 triiodo L thyronine can be synthesized almost as readily as thyroxine it seemed to us important to test its activity by the galactopoietic response in cattle since if it proved several times more active in this respect than thyroxine the cost of the treatment might be considerably lowered However, in feeding experiments in lactating cows in which the effects of three doses of 3 5 3 triiodo L thyronine were compared with that of a dose of 75 mg synthetic L-thyroxine daily we found that even the highest dose of the former 64 mg daily, had very little effect on milk secretion (Bartlett, Burt, Folley and Rowland 1954) This is evident from Figure III 14 which shows the mean milk yield responses of the various groups of cows used in this experiment Each curve shows the increase in the milk yield of a group of cows over the mean value for the group before feeding began and is corrected for the change in milk yield undergone by a comparable group of untreated control cows The effects of the treatments on the milk phosphatase levels led to similar conclusions

This suggested three alternative possibilities, (a) that 3 5 3 triiodo L-thyronine has relatively little inherent galactopoietic activity, (b) that it is not well absorbed from the digestive tract of the cow or (c) that it is more readily

inactivated in the rumen than thyroxine The first possibility was studied in an experiment in which the galactopoietic effects of equal doses, 5 mg daily, of the two substances given

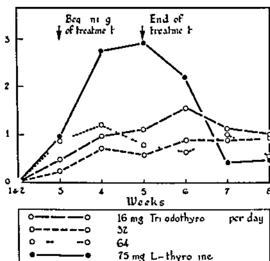



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crease in the enzyme concentrations than the same amount of L thyroxine However, in view of the relatively slight activity of 3 5 3 triiodo L thyronine by the oral route in cows (the only useful one for the farmer), which we believe to be due to its rapid inactivation by rumen microorganisms, this substance clearly has no practical possibilities for use in cattle

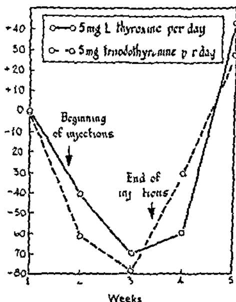



Fig III 15 Percentage change in milk phosphatase in the cow after subcutaneous injections of L-triiodo thyronine *(from Bartlett Burt Folley and Rowland 1953)*

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# CHAPTER IV

# THE PHYSIOLOGY OF SUCKLING AND MILKING

## THE MILK EJECTION REFLEX

It has long been known that the withdrawal of milk from the mammary gland whether by the suckling young, the hand milker or the milking machine, involves the active participation rather than the passive acquiescence of the lactating animal In speaking of the active participation of the mother I do not mean her behaviour pattern in promoting access of the young to her nipples which we may call nursing behaviour * I use the word participation in a limited sense to mean the role of the lactating animal in promoting the flow of milk from the mammary tissues a role which is quite unconscious

The greater part of the milk present in the fully charged mammary gland, which in essence comprises the contents of the alveoli and finest ducts, only becomes available for transfer through the teat to the exterior of the gland if forcibly expressed from the alveoli into the larger ducts by the reflex contraction of effector contractile cells forming a network over the stromal surface of the alveoli This reflex the existence of which has been tacitly recognised for some time (see Folley 1947 for review), manifests itself by a sudden rise in the milk pressure in the mammary gland in response to the stimulation of sensory nerve endings in the teat that is the application of the suckling or milking stimulus This sudden reflex rise in intra mammary pressure is illustrated in Figure IV 1 The figure taken from a paper by Tgetgel (1926) shows the curve of increase of milk pressure in the udder cistern of a cow

* Cowie Folley Cross Harris Jacobsohn and Richardson (1951) have proposed a system of terminology for use in lactational physiology this terminology is adopted in this chapter (see also Cowie Folley and Richardson 1954)

between one milking and the next We see how two successive applications of the milking stimulus at times marked by the arrows, caused sudden temporary increases in the pressure of milk in the gland cistern which soon subsided even though no milk was withdrawn The milk ejection reflex is the means by

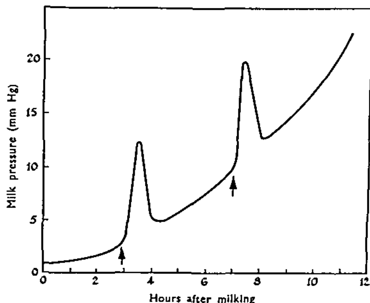



Fig IV 1 Milk pressure curve between one milking and the next (cow) reaction to the milking stimulus *(from Tgetgel 1926)*

which the active but unconscious participation of the lactating mother in the act of nursing, essential if the full yield of available milk is to be obtained, comes into play Plate IV 2 shows a famous painting by the Venetian master, Tintoretto It is called The Origin of the Milky Way' and hangs in the National Gallery in London It seems to have been inspired by a classical myth, one version of which tells how Jupiter descended from Olympus to pluck the infant Hercules from the breast of Juno The picture is of interest in the present connection because it illustrates some significant points about the essential nature of the milk-ejection reflex It is as if Tintoretto, if not the ancients intuitively knew that the suckling stimulus causes some response in the lactating breast

which results in a tendency for its contents to be forcibly expelled Another important point illustrated by the painting is that the response to suckling is systemic rather than local, for the baby suckles one breast but milk is ejected from both (see Newton and Newton, 1948) In this connection we may note that it is the exception rather than the rule for milk to spurt from the nipple when the milk ejection reflex operates unless the resistance of the teat sphincter is overcome by the mouth of the suckling or the action of the teat cup of the milk ing machine

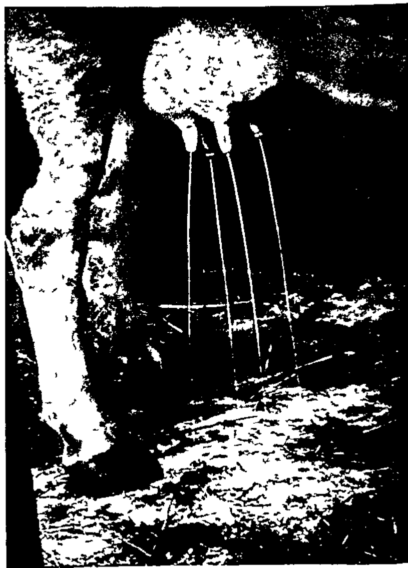
The importance of this reflex for the total phenomenon of lactation is obvious If it fails to occur the end result will be the same as if secretion itself had ceased It is for this reason that it is difficult to interpret many experiments in which the effects upon lactation of the section of afferent nerves from the mam mary gland have been studied The rather elegant experiments of Ingelbrecht (1935) have often been cited He denervated the abdominal mammae of lactating rats and showed that the young died from lack of milk if they were prevented from suck ling the thoracic glands while being allowed access to the ab dominal nipples If however suckling of even two of the tho racic glands was permitted then the young could obtain milk from the abdominal glands and the whole litter thrived Now there is evidence that the suckling stimulus not only evokes the milk ejection reflex but also reflexly excites the secretion by the anterior pituitary of lactogenic hormones which main tain the mammary tissues in a secretory condition It is thus not clear whether Ingelbrecht was concerned with a failure of milk secretion or with a block in the milk expulsion mechanism or both Partial failure of the reflex because of faulty prepara tion for milking may be of practical importance for if habitual it could result not only in serious malnutrition of the sucklings but also in shortening of the lactation period resulting from acceleration of involution of the mammary alveolar tissue because abnormal amounts of secretion are retained In this connection it may be noted that milking does not completely empty the alveoli of secretion as evidenced by the fact now well known that injection of posterior pituitary extract immediately after milking enables one to obtain some more milk especially rich in fat

Like other reflexes the milk ejection reflex can be conditioned It is a matter of common observation that in the cow, it easily becomes conditioned to a variety of auditory and visual stimuli, associated with milking shed routine, such as the rattling of milk buckets, washing the udder, etc Waller (1938) in his well known book, relates examples of conditioning in lactating women One woman was accustomed to drink a glass of water before putting her baby to the breast, soon the mere act of filling the glass was sufficient to evoke the reflex Like other conditioned reflexes it can be inhibited by disagreeable, emotionally disturbing stimuli as has been shown experimentally by Ely and Petersen (1941) and Whittleston (1951) in the cow, Newton and Newton (1948) in woman and Cross (1955*b*) in the rabbit It seems likely that this inhibition is central in most instances though in others it may be mainly peripheral (see Cross 1955*b*) The probability that activation of the sympathetico adrenal system may often be responsible for the inhibition is indicated by the fact that as shown by Ely and Petersen (1941), Cross (1953, 1955*a*) and others, adrenaline will block the normal reflex As we shall see later, the milk ejection response can be evoked by certain experimental procedures such as the injection of oxytocin and electrical stimulation of the supraoptico hypophysial tract Adrenaline injection will interfere with these responses also For instance, Braude and Mitchell (1952) have shown that the milk ejection response to oxytocin in the lactating sow, which some claim to be especially clear cut, can be almost completely blocked by the prior injection of adrenaline In the rabbit, Cross (1953, 1955*a*) has shown that adrenaline will block the milk ejection response both to oxytocin and to electrical stimulation of the supraopticohypophysial tract These observations perhaps suggest that the inhibitory effect of adrenaline is peripheral, that is, that it abolishes the effect of the milk ejection hormone in the mammary gland itself In fact, Cross (1955*a*) believes that the inhibitory effect of central stimulation on the milk ejection reflex may depend on the constriction of mammary blood vessels, thus impeding access of oxytocin to the tissues The fact that Linzell (1955) found that adrenaline did not abolish the alveolar contracting effect of topically applied oxytocin on the mammary gland

PLATE IV 2



*Reproduced by courtesy of the Trustees, The National Gallery, London*



Milk gushing from the udder of a cow whose teats have been cannulated after an intravenous injection of 180 i u oxytocin (*from Usuelli and Piana* 1952)

of the living mouse is in accord with this Cross (1953 1953a) further showed that endogenous adrenaline released in response to electrical stimulation of sympathetic centres in the posterior hypothalamus also inhibited the milk ejection response to oxytocin In view of all this evidence we may conclude that activation of the sympathetico adrenal system can inhibit the milk ejection reflex and that this inhibition is probably though not necessarily exclusively peripheral This mechanism is almost certainly not involved in all cases of inhibition since the latter may be induced by stimuli such as embarrassment (Newton and Newton, 1948) which probably do not activate the sympathetico adrenal system The experiments of Cross (1955b) on the rabbit led him to conclude that the main factor in the inhibition due to emotional disturbance is interference with the release of oxytocin by the posterior pituitary

EVIDENCE FOR THE NEUROENDOCRINE REGULATION OF MILK EJECTION

Let us now proceed to consider evidence that the milk ejection reflex is neuroendocrine in nature

The milk ejection reflex was once believed to consist of a purely neural arc This belief is however no longer held there being abundant evidence as will be shown later that the arc is a neurohormonal one The efferent component of this arc is the release from the neurohypophysis into the blood stream of a factor which as we shall see later is probably oxytocin and which is believed to evoke the contraction of the aforementioned effector tissue associated anatomically with the mammary alveoli No single piece of evidence for this theory is conclusive by itself but there is now such a coherent body of concurrent and harmonious findings from various angles that the neuroendocrine theory of milk ejection can be accepted as in principle well established Nevertheless details of the afferent pathways and the precise chemical nature of the hormonal component and the identity of its target tissue in the mamma remain to be further investigated

It has been known for over forty years ever since the pioneer work of Ott and Scott (1911) followed a little later by that of Schafer (1913) that the milk ejection reflex can be mimicked by the injection of posterior pituitary extract Plate IV 3

from Usuelli and Piana (1952) shows how an injection of oxytocin into a lactating cow whose teats have been cannulated causes the milk to gush forth It is forty years since Gaines (1915) described interesting experiments which today would unhesitatingly be interpreted as providing cogent evidence for a physiological role for the neurohypophysis in milk ejection Among other interesting findings, he showed that in the bitch the reflex could be inhibited by ether anaesthesia and the inhibition overcome by the injection of posterior pituitary extract These facts are illustrated in Figure IV 4 taken from Gaines (1915) which shows the milk flow curves

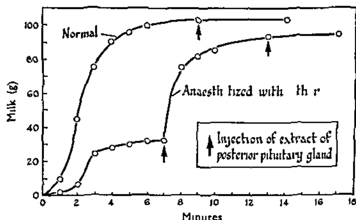



Fig IV 4 Milk flow curves for a bitch nursing puppies *(from Gaines 1915)*

for a lactating bitch obtained by weighing her puppies at intervals during suckling It will be seen that when the mother was anaesthetized the puppies could get milk at first but the flow soon stopped This represents the milk present in the larger sinuses which, like that contained in the cisterns of the bovine udder, can be drawn off without the intervention of the milk ejection reflex * If after the initial flow had ceased the bitch was given an intravenous injection of posterior pituitary extract the milk began to flow again and the puppies were able to empty her breasts Essentially similar findings in the rabbit were reported by Cross (1955*b*) forty years later

* i e. passive withdrawal (see Cowie *et al* 1951)

Despite his clear cut and significant results Gaines regarded the milk ejection reflex as a purely neural arc probably because hardly anything was known of neuroendocrine relationships at that time so that there was no background of knowledge which would have led anyone to conceive a physiological role for the posterior lobe in this function The position remained substantially unchanged until about 1940 when Ely and Petersen (1941) performed experiments which prompted them to advance for the first time the theory that the suckling or milking stimulus causes reflex secretion by the posterior pituitary of a hormone believed by them to be oxytocin, which in turn evokes contraction of an effector tissue capable of expressing milk from the mammary alveoli Ely and Petersen (1941) showed *inter alia* that after section of two nerves believed to carry all the motor fibres to one half of the udder of a cow the milk ejection response could be induced by the milking stimulus or oxytocin injection in normal and denervated udder halves alike Moreover the reflex response to the milking stimulus could be blocked in both udder halves by adrenaline or by fright induced by sudden, loud noises Provided one can feel satisfied that the operative procedures had indeed cut all efferent fibres to the udder and none had regenerated at the time of the experiment, these findings argue against motor innervation of the effector contractile tissue of the udder and suggest that the terminal efferent link is hormonal in nature

Later, more direct evidence that the milking stimulus evokes the liberation into the blood stream of a hormone which causes milk ejection was afforded by experiments in which it was shown that if blood taken from a cow immediately after the application of the milking stimulus is perfused through an isolated bovine udder with teats cannulated there is a more rapid and copious flow of milk than occurs when blood from a non stimulated cow is used Table IV 1 shows some results obtained by Peeters Massart and Coussens (1947) by means of their perfusion technique which permits the simultaneous but independent perfusion of the two separated halves of a bovine udder The milk flow from the udder half perfused with blood taken from a cow after she had been stimulated to eject her milk was in all cases greater than the flow from the

control udder-half perfused with blood from an unstimulated cow These results are essentially similar to those earlier reported by Petersen and Ludwick (1942)

TABLE IV I

*Expulsion of milk from the two halves of a bovine udder perfused respectively with blood from the same cow before and after application of the milking stimulus*

(from Peeters Massart and Coussens 1947)

| Experiment no | Amount of milk expelled from the udder tissue soon after circulation was established | |
|---|---|---|
| | Left half perfused with blood taken before milking stimulus applied ml. | Right half perfused with blood taken after milking stimulus applied ml. |
| 1 | 160 | 360 |
| 2 | 270 | 520 |
| 3 | 215 | 345 |
| 4 | 390 | 500 |

A number of experimenters have provided evidence that the milk ejection reflex involves activation of the posterior lobe of the pituitary gland by demonstrating the occurrence of an antidiuretic effect of the posterior pituitary type shortly after the application of the suckling or milking stimulus to a water loaded lactating subject Such results have been obtained in the rabbit by Cross (1950), the bitch by Kalliala, Karvonen and Leppanen (1952), the cow by Peeters and Coussens (1950) and in woman by Kalliala and Karvonen (1951) Now much work, mainly biological but culminating with the chemical work of du Vigneaud who with his colleagues prepared two highly purified posterior lobe polypeptides which are biologically active (see du Vigneaud 1952) strongly suggests that the antidiuretic and vasopressor principles of the posterior lobe are identical Moreover, P[illegible] d Coussens (1950) found that, in the cow mild [illegible] inhibited the antidiuretic response

without interfering with the milk ejection reflex Therefore, we may conclude that while the experiments we have been considering provide good evidence that the milking stimulus activates posterior pituitary functions it would seem improbable that vasopressin is the natural milk-ejection hormone though, as we shall see, posterior pituitary vasopressor fractions undoubtedly possess some milk ejection activity

We thought it might be interesting to approach the problem in another way It seemed possible that the milking stimulus might cause a measurable loss of oxytocic or vasopressor activity from the posterior lobe Accordingly Mrs M H I Dodd, in our laboratory, made careful measurements of the content of both activities in the posterior pituitary glands of goats some of which were autopsied just after milking while

TABLE IV2

*Amounts of oxytocin and vasopressin in posterior pituitaries of goats*

(unpublished work of Dodd cited by Folley 1952)

| No of goat | Sex | Age | Vasopressin i u /mg dry tissue | Oxytocin i u /mg dry tissue |
|---|---|---|---|---|
| *Non lactating goats* | | | | |
| 393 | ♀ | 6 months | 0 7–0 8 | 0 8 |
| 385 | ♀ | 8 ,, | 1 8 | 1 8 |
| 342 | ♂ | 2½ years | 0 5 | 0 6 |
| *Lactating goats, unmilked for 24 hr before autopsy* | | | | |
| 316 | ♀ | 2 years | 0 6 | 0 8 |
| 326 | ♀ | 2 ,, | 0 6 | 0 6 |
| 338 | ♀ | 2 ,, | 0 8 | ≏ 0 7 |
| 100 | ♀ | 6½ | 0 8 | 0 8 |
| *Lactating goats, killed immediately after milking out* | | | | |
| 79 | ♀ | 8 years | 0 7 | 0 65 |
| 127 | ♀ | 6 ,, | ≏ 0 6 | ≏ 1 0 |
| 165 | ♀ | 5 | 0 8 | 0 8–1 0 |
| 291 | ♀ | 3 | 0 6 | 0 8 |
| 232 | ♀ | 4 ,, | 0 8 | 0 8–1 0 |
| 222 | ♀ | 4 ,, | 0 6 | 1 0 |
| 226 | ♀ | 4 | 0 28(?) | 0 7 |

others had gone unmilked for some hours before autopsy (unpublished work by Dodd, 1951, cited by Folley, 1952) However no depletion of biological activity due to milking could be demonstrated As will be seen from the results given in Table IV 2 she found that the absolute amounts of oxytocic and vasopressor activities present in the posterior lobes of goats shot immediately after normal milking were the same as those of goats unmilked for 24 hr before autopsy Moreover, the values for a small series of dry goats were indistinguishable from those of lactating animals In all cases save one the concentrations of both principles, measured in international units were equal, as they are by definition in the international standard ox posterior pituitary powder Thus the milking stimulus caused no measurable alteration in the ratio of the concentrations of vasopressin and oxytocin in the posterior pituitary gland Similarly, Whittlestone, Bassett and Turner (1952) assaying posterior lobes from cows for milk-ejection activity by a special technique depending on the stimulation of milk flow in the lactating sow could find no unequivocal evidence of any appreciable decrease in this activity in the pars nervosa as a result of normal milking It may be that the amounts of posterior-lobe activities discharged in response to the milking stimulus are too small in relation to the amounts stored in the lobe to enable the difference to be detected by assay methods at present available In our laboratory, Dr A T Cowie has found that an intravenous injection of 1 i u oxytocin into an anaesthetized lactating goat will give a milk ejection response approximating in magnitude and duration to that obtained during normal milking (unpublished work of Cowie 1951 cited by Folley, 1952) His results are illustrated in Figure IV 5 which shows kymograph tracings of the rise in intra mammary pressure evoked by different in travenous doses of oxytocin into an anaesthetized goat with one teat cannulated and the cannula connected to a pressure recording device Goat posterior lobes contain 10–15 i u oxytocin (personal communication from Mrs M H I Dodd) so that the milking stimulus may cause only a discharge of 10 % or less of the amount stored in the pars nervosa The discharge may be much less even than this according to results recently reported by Denamur and Martinet (1953)

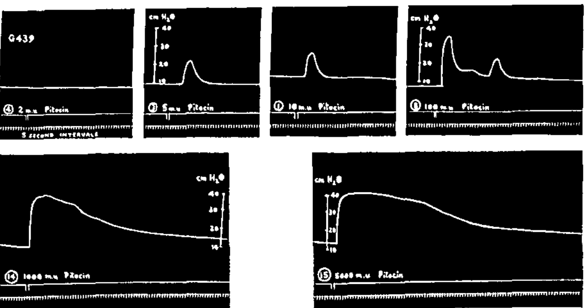



Fig IV 5 Pressure changes in the cistern of the mammary gland of a goat after intravenous injection of oxytocin *(from Cowie unpublished results cited by Folley 1952)*

These authors obtained good milk ejection responses in anaesthetized goats receiving 1 mu oxytocin intravenously Maximal responses were given by 10 mu and, in some animals, just detectable effects with only 0 1 mu!

The latest and most convincing evidence of the intervention of a hormone of the neurohypophysis in the milk ejection reflex comes from experiments involving electrical stimulation of the hypothalamus In the anaesthetized rabbit Cross and Harris (1951, 1952) have shown that electrical stimulation of the supraoptico hypophysial tract caused ejection of milk from a cannulated teat duct Effects of similar magnitude, measured with a manometer recording on a kymograph, could be elicited by the intravenous injection of 200 mu posterior pituitary extract Rabbits in which electrolytic lesions had previously been placed in the supraoptico hypophysial tract, so that the neurohypophysis distal to the lesion had presumably degenerated, failed to exhibit the milk-ejection reflex in response to electrical stimulation of the tract, nor could their litters obtain significant quantities of milk in suckling tests The young could only withdraw milk from these animals if 30–200 mu posterior pituitary extract were intravenously injected immediately before suckling began In those cases where subsequent histological examination showed that the hypothalamic lesions had missed the supraoptico hypophysial tract, suckling by the young or electrical stimulation of the tract elicited a normal milk ejection response The long latent period, which outlasts the stimulus and the persistence of the effects after the stimulus has ceased are good evidence that Cross and Harris were dealing with a neuroendocrine mechanism involving the neurohypophysis

Similarly, Andersson (1951*a*) has evoked milk ejection responses in unanaesthetized lactating ewes and goats by electrical stimulation of hypothalamic centres in or adjacent to the supraoptic nuclei Andersson concluded that these responses were undoubtedly hormonally mediated because they could be evoked during sacral anaesthesia or in denervated udder halves Moreover, he showed that blood taken from a goat immediately after electrical stimulation evoked a prompt flow of milk from the cannulated teats of another lactating animal on intravenous injection This indicates that




X ray photograph of the head of a goat showing electrodes implanted in the hypothalamus (*photograph by courtesy of Dr B Andersson*)

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the stimulation had caused the discharge of a milk-ejection hormone into the blood stream Andersson s experiments were made by the technique of Hess and Plate IV 6 shows an X ray picture of the electrodes in position in the brain of one of his goats

Experiments on section of the pituitary stalk have not con tributed much to our knowledge of the milk ejection reflex In cases where stalk sectioned animals were found to rear their young it is possible that the milk ejection hormone came from that part of the neurohypophysis proximal to the point of section This might explain why the parturient neurohypo physectomized animals, described many years ago by Smith (1932) and by Houssay (1935), reared their young However in our laboratory, Dr A T Cowie has found that posterior lobectomized rats possessing functional anterior lobe tissue so that secretion was maintained would not rear their young unless milk ejection was induced two or three times daily

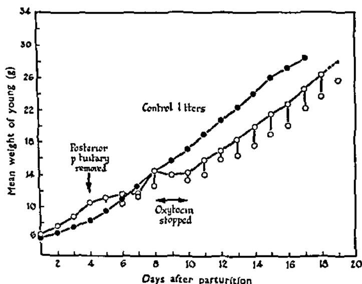



Fig IV 7 Growth curve of the litter of a rat neurohypophysectomized (posterior lobectomy) on the 4th day of lac tation and injected three times daily with oxytocin *(from Cowie unpublished results cited by Folley 1952)*




Thick section (75 μ) of part of a lobule from a goat mammary gland fixed when distended showing the myoepithelial cells stained with silver (*from Richardson* 1949)

can be accounted for by its contamination with oxytocin, stated by the manufacturers (Parke, Davis & Co ) to amount to no more than 5 %

Turner and Cooper (1941) suggested as alternative explanations of their results either that milk ejection activity is a property both of oxytocin and vasopressin or else that the milk ejection hormone is a distinct and separate posterior lobe principle Andersson (1951*b*) however, attempted to explain the discrepancy by assuming that a small amount of oxytocic activity is inherent in vasopressin itself and that oxytocic and milk ejection activities are related biological properties of the same posterior pituitary hormone He suggested that if after an injection of oxytocin or vasopressin equal amounts of either disappear from the blood in a given time the proportional destruction of the small amount of milk ejection activity associated with a relatively large amount of vasopressin would be less than of that associated with a relatively small amount of oxytocin According to this view the discrepancy arises from the fact that oxytocic activity is usually measured *in vitro* (by its effect in eliciting contractions of strips of uterus) while milk ejection activity is measured *in vivo* by a systemic response The results of Whittlestone (1952) who found that various oxytocic preparations possess milk ejection activity in proportion to their oxytocic activities while vasopressin preparations including du Vigneaud s highly purified vasopressin polypeptide exhibited more milk ejection activity than could be accounted for by their oxytocic activities, suggest that some milk ejection activity is inherent in the vasopressin molecule A similar conclusion follows from the work of Cross and van Dyke (1953) who studied the milk ejection activities of du Vigneaud s highly purified oxytocic and vasopressor polypeptides in the rabbit

I suggest that we need not attach undue importance to the discrepancy between the milk ejection and oxytocic activities of vasopressin preparations for the following reason Coon (1939) has described a method for the assay of oxytocic activity depending on the fall in blood pressure of the anaesthetised fowl When posterior pituitary extracts in which vasopressin predominates are assayed by this method the oxytocic activities found are greater than are those given by

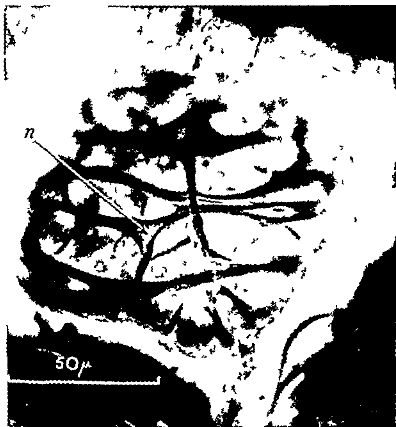
by the injection of oxytocin thus allowing the young regular suckling periods (unpublished work of Cowie, 1951, cited by Folley, 1952) Figure IV 7 illustrates his results The black points represent the growth curve of the young of a group of control rats, the white circles the growth curve of the litter of a rat neurohypophysectomized on the fourth day of lactation and thereafter given oxytocin three times daily The vertical lines represent the amounts of milk obtained by the litter each day following the first injection of oxytocin into the mother It will be seen that the young of the neurohypophysectomized rat grew at approximately the normal rate A significant point to be noted is the temporary, but immediate, cessation of growth of the young when oxytocin was withheld from the mother on the 9th day The elegant experiments of Harris and Jacobsohn (1952) are also illuminating in connection with the effects of severance of the pituitary stalk upon milk ejection They showed that the mammae of hypophysectomized rats possessing functional anterior pituitary grafts, that is grafts in which the hypophysial portal circulation was re established, contained milk but the animals would only rear their young if regularly injected with posterior pituitary extract

It is evident from what has been said that the evidence for the neuroendocrine regulation of milk ejection by a pathway involving activation of the posterior pituitary is now impressive in amount and in sum convincing

### NATURE OF THE MILK EJECTION HORMONE

Let us now consider the nature of the milk ejection hormone

It has uniformly been found that while both the oxytocic and vasopressor fractions from posterior lobe extracts possess milk ejection activity, the oxytocic fraction is much more active in this respect than the pressor fraction This has given rise to the belief, now held by most workers in the field that the oxytocic principle is the natural milk ejection hormone However, the activity of the vasopressor preparation, Pitressin has been found by Turner and Cooper (1941), Cross and Harris (1952) and Andersson (1951*b*) to be about 20 % of that of the oxytocic preparation, Pitocin, which is much greater than

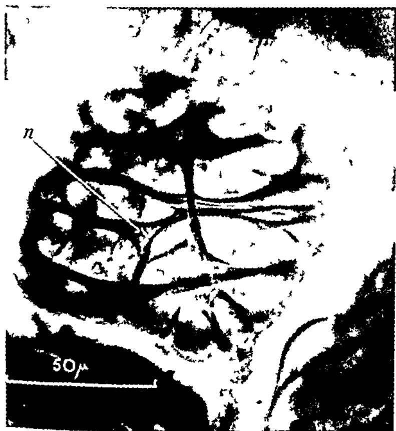



Photograph of part of the surface of a small contracted alveolus (goat) showing a myoepithelial cell with nucleus (*n*) and branching processes (*from Richardson* 1949)

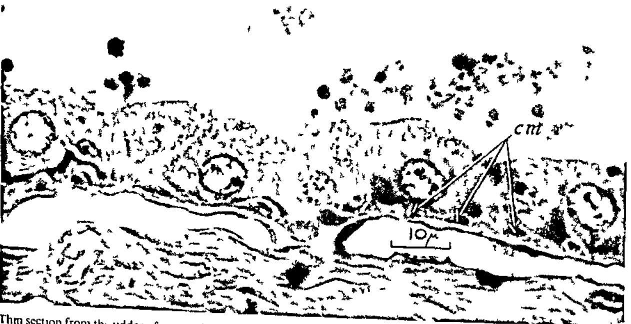
the conventional method depending on the contraction of uterine muscle *in vitro* Thus du Vigneaud (1952) has reported that the oxytocic activity of his purified vasopressor polypeptide came out three times larger by the chicken blood pressure method than by the uterine muscle assay Coon thought it possible that when present in excess, vasopressin may interfere with the uterine muscle response *in vitro* so that oxytocic activities are underestimated by the *in vitro* technique on the other hand the results of Strahan and Waring (1954) indicate that the chicken blood pressure technique may over estimate oxytocic activity in some circumstances The point is that available methods for oxytocin assay seem to be subject to uncertainties at the present time

In this connection assay values for highly purified oxytocin and ADH vasopressin polypeptides given in their Table I by van Dyke, Adamsons and Engel (1955) are of interest, for in the case of ADH vasopressin, assays based on the milk ejection response in the rabbit and the depression of the blood pressure in the fowl were in substantial agreement while a much lower value was given by the rat uterus method, with oxytocin, however identical values were given by all three methods

The most reasonable explanation of the available evidence seems to be that milk ejection and oxytocic activities are facets of the same biological activity characteristic of the oxytocic polypeptide and that both activities are possessed in some small degree by the vasopressor polypeptide also It is unlikely that vasopressin is the natural milk ejection hormone because Peeters and Coussens (1950) in the cow and Cross (1950) in the rabbit found that the antidiuretic response in lactating animals could be elicited by the injection of amounts of posterior pituitary extract too small to cause milk ejection Moreover, as previously pointed out mild fright will inhibit suckling antidiuresis in lactating cows without blocking the milk ejection reflex (Peeters and Coussens 1950) Finally in the present connection it is worth noting that du Vigneaud, Ressler, Swan, Roberts Katsoyannis and Gordon (1953) have described the synthesis of a polypeptide possessing oxytocic activity and also the power to evoke milk ejection




Photograph of part of the surface of a small contracted alveolus (goat) showing a myoepithelial cell with nucleus (*n*) and branching processes (*from Richardson* 1949)




Thin section from the udder of a goat showing the processes of the *myoepithelial* cells cut transversely and appearing in the form of oval or triangular bodies (*c m*) (*from Richardson* 1949)

THE EFFECTOR CONTRACTILE TISSUE OF THE MAMMARY GLAND

In conclusion, we will consider briefly the nature of the effector contractile tissue of the mammary gland This aspect of the milk ejection reflex has been considerably clarified as a result of recent work Many authors had until even quite recently regarded smooth muscle tissue as the contractile agent, which they had supposed, on rather inadequate evidence, to occur in the mammary gland in close association with the mammary alveoli in sufficient abundance to play an effective physiological role in squeezing milk from the alveolar tissues Seven years ago, Richardson (1949*a*), in a careful histological study of the mamma of the goat found, however that quantitatively there is not nearly enough smooth muscle tissue surrounding the lobules to be effective in causing contraction of the alveoli individually or of the lobules as a whole

Earlier histologists had figured myoepithelial cells or basket cells in close association with the mammary alveoli, as cells of ectodermal origin which by analogy with smooth muscle cells were assumed to be contractile However there was subsequently some reluctance to accept these cells as the effector contractile tissue because until Richardson (1949*a*) had devised a satisfactory technique for visualising them by silver impregnation their morphological characteristics quantitative distribution and concentration had been vague Richardson s studies, first on the goat (1949*a*) and later on the human breast (1951) have however shown conclusively that myoepithelial cells are present throughout the mamma in the closest association with the duct and alveolar epithelia They lie on the epithelial side of the basement membrane and their branching processes form a network over the stromal alveolar surface – hence the appositeness of the name basket cells Plate IV 8 for which, like the remainder of the illustrations in this chapter, I am indebted to Mr K C Richardson shows a thick section (100$\mu$) of alveolar tissue from a goat udder Thick sections are best for demonstrating myoepithelium because fields can be chosen in which portions of the alveolar walls are shown in surface view In this figure the branching processes of the myoepithelial cells blackened with silver are clearly visible Plate IV 9 shows part of the

surface of one alveolus at a higher magnification and gives an excellent impression of the myoepithelial network with the nucleus of one of the myoepithelial cells near the centre of the field It is evident from these brilliant photomicrographs of Richardson that quantitatively these cells are sufficiently abundant – much more so than had formerly been realized – to qualify them for the collective role of effector organ in the milk ejection reflex The observations of Richardson on the occurrence of myoepithelium in the mammary gland of the goat and woman have since been confirmed in the cat and other species by Linzell (1952) It seems probable that the smooth muscle" cells described by Swanson and Turner (1941) fifteen years ago in *thin* sections of mammary alveolar tissue were in reality the myoepithelial cells of Richardson which, as has been pointed out, can best be studied in thick sections In thin sections it is only possible to see, as shown in Plate IV 10 the branching processes of the myoepithelial cells in cross section

As regards the probable role of these cells in the milk ejection reflex, Richardson (1949*a*) has shown they change their configuration when the alveoli collapse as a result of milking This is illustrated in Plate IV 11 The two upper pictures show sections of alveolar tissue from the two halves of a goat udder the left hand one being from an udder half fixed in a condition of pre milking distension and the right from an udder half fixed in a collapsed condition just after milking out The change in shape and orientation of the myoepithelial cells following milking is clearly shown in the corresponding photomicrographs set out beneath As a result of careful study of these morphological changes, Richardson (1949*a*) concluded that the myoepithelial cells assume an orientation in relation to the folds of the alveolar epithelium in the post milking contracted ' gland which is consistent with the view that the wrinkling of the alveolar wall is due to their active contraction rather than with the idea that they passively conform to the contours of the alveoli as the latter collapse Myoepithelial cells also occur in abundance next to the stromal surface of the duct epithelium, in this case their contraction would tend to widen and shorten the ducts thus reducing the resistance to the passage of milk and facilitating its escape











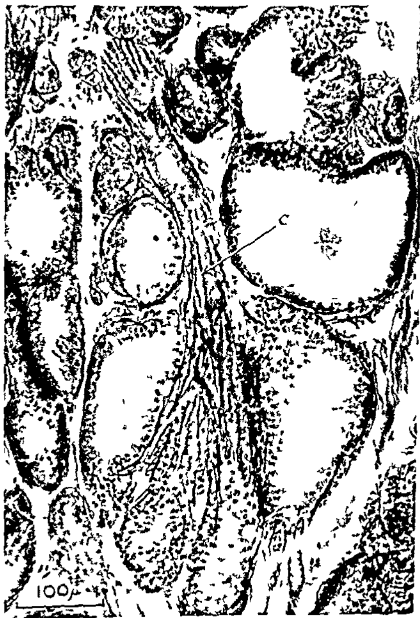

Comparison between distended and contracted glands (goat) —

*Top left* part of a lobule from the left half of an udder fixed while distended with milk

*Top right* The right half of the udder from the same goat which was milked out as completely as possible before autopsy note the contracted lobules with collapsed alveoli and ducts lined with a thick folded epithelium

*Bottom left* Myoepithelium on the surface of distended alveoli section taken from the same left half as above

*Bottom right* Myoepithelium on contracted alveoli section taken from the same right half as above (*from Richardson* 1949)




Longitudinal orientation of myoepithelium on an intra lobular duct (*c*) connected with some large elongated alveoli (*from Richardson* 1949)

from the alveolar tissue Plate IV 12 shows a thick section from a goat mammary gland which includes parts of the outer wall of a milk duct in surface view The longitudinal orientation of the silver blackened myoepithelial cells is clearly seen

There can be little doubt that the effector contractile tissue of the mammary gland is the myoepithelium which must play the major role in the active expulsion of milk from the alveolar tissue though there remains the possibility that vasomotor effects may also play some part The evidence though good is still only circumstantial, however and the matter cannot be regarded as settled beyond all possible doubt until it is possible to devise a technique for observing directly in living mammary tissue, the contraction of the myoepithelium experimentally induced A step towards this desirable end has recently been made by Linzell (1955) who has observed under the microscope the contraction of mammary alveoli in the living mouse in response to the local application of oxytocin and vasopressin preparations Richardson (1949*b*) has pointed out that until more is known of their pharmacological responses innervation and other properties it seems desirable to continue to regard the myoepithelial cells and smooth muscle of the mammary gland as separate physiological entities

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# CHAPTER V

# THE BIOSYNTHESIS OF MILK FAT AND ITS HORMONAL CONTROL

## EARLIER THEORIES OF THE ORIGIN OF MILK FAT

Of the three major milk constituents, casein fat and lactose most is so far known about the biosynthesis of milk fat and this chapter will be devoted to consideration of this topic The mammary gland is very active in the synthesis of fat, so much so that it is surprising that this tissue has until recently been comparatively neglected by biochemists interested in lipo genesis* Nevertheless recent studies of fat synthesis in the mammary gland have contributed materially to our know ledge of lipogenesis in general as I shall hope to show

Let us begin by discussing the various views that have been held at different times about the nature of the precursors of milk fat which are brought to the mammary gland in the blood Many years ago Meigs Blatherwick and Cary (1919) advanced the theory that the precursor of milk fat was the phospholipid fraction of the blood lipids They pointed out that if this were true the lactating udder would take up more than enough phospholipid phosphorus to provide the inorganic phosphate of the milk and they believed that their experimen tal results constituted valid evidence for this theory since they found that the jugular blood of lactating cows contained more inorganic phosphate than the blood leaving the udder in the mammary vein However, subsequent investigations (Lintzel 1934 Graham Jones and Kay, 1936) in which the composition of arterial blood was compared with that of blood from the mammary vein of the lactating cow failed to provide evidence of any uptake of phospholipid phosphorus by the udder and moreover there was general agreement that arterial blood

* For that matter the same remarks apply equally to the synthesis of protein and carbohydrate

contained more inorganic phosphate than mammary venous blood The hypothesis of Meigs already thus contra indicated was finally disproved by Aten and Hevesy (1938), who in one of the earliest applications of radioactive phosphorus to bio chemical studies showed that the inorganic phosphate of the blood plasma was the direct precursor of the inorganic phos phate of the milk

Meanwhile the e same arterio venous studies (Lintzel, 1934 Graham *et al* 1936, Maynard, McCay, Ellis, Hodson and Davis, 1938) had shown that the only fraction of the blood lipids which undergoes significant diminution in passage through the lactating udder is the neutral fat fraction, that is the glycerides and possibly the cholesteryl esters, and attention accordingly became focussed on the blood glycerides as the precursors of the milk glycerides

It is necessary at this juncture to point out that the glyceride structure of milk fat exhibits certain characteristic features which distinguish it from fats found elsewhere in the animal body The milk glycerides particularly of herbivorous ani mals, contain considerable molar proportions of short chain saturated fatty acids, $C_4$–$C_1$ , which are not found in animal depot fats It is clear that any acceptable theory of the bio synthesis of milk fat must account for the presence in milk fat of these short chain acids and not unnaturally there has been much speculation about their origin

The theory that the milk fat arises directly from the blood glycerides, which has held the field until quite recently, was supported by two main lines of evidence On the one hand was the evidence from arterio venous studies carried out on the udder of the lactating cow which has already been mentioned All these studies showed that there is an appreciable uptake of neutral blood fat by the lactating bovine udder and some authors, notably Shaw and Petersen (1938), have further claimed that the uptake of blood fat is more than enough to provide the fat of the milk These workers were thus led to postulate that some of the fatty acids of the blood glycerides absorbed by the udder were partially degraded by oxidative processes in the lactating gland, the resulting fatty acid fragments representing the short chain acids of the milk glycerides This idea of the origin of the milk short chain fatty acids was in

harmony with the theory previously advanced from quite a different viewpoint by Hilditch (1937) whose arguments were based on extensive and painstaking chemical investigation of the glyceride structure of milk fat (see also Achaya and Hilditch, 1950) To discuss the outstanding work of Hilditch and his collaborators on the chemistry of milk fat in sufficient detail to do justice to the way in which it has advanced this branch of chemistry is outside the scope of this chapter All that can be said in the limited space available is that these studies revealed the existence of interesting relationships between the proportion of the milk glycerides which contain *only saturated fatty acids and the proportion of saturated fatty* acids in the total acids relationships which were of a type characteristic of milk fats and animal depot fats alone among natural fats Briefly Hilditch and his colleagues found that wherever a short chain acyl residue occurred in a glyceride molecule from milk fat one would have expected to find an oleic residue had the glyceride been a component of a body fat These quantitative relationships led Hilditch to deduce that long chain fatty acids particularly oleic acid present in the blood glycerides are broken down in the udder by oxido reduction processes thus giving rise to the short chain acids of the milk fat and also to the unsaturated fatty acids of the $C_{10}$–$C_{16}$ series containing a double bond in the same position as in oleic acid which occur in traces in cow s milk

Many observations in addition to the aforementioned arterio venous differences in blood neutral fat across the mammary gland are in harmony with the theory of Hilditch As an example may be cited the decrease in the molar proportion of short chain acids in the milk fat, accompanied by a concomitant increase *in the proportion of oleic acid caused by* inanition (Smith and Dastur, 1938) or by feeding cod liver oil (Hilditch and Thompson, 1936) However none of the evidence that the short chain acids of the milk fat are formed by oxidative *degradation of long chain acids arising from the* blood glycerides is conclusive, although there is no reason to challenge the conclusion which follows from the arterio venous studies that the lactating udder takes up appreciable quantities of *neutral fat from the blood* Presumably some portion at least of this blood fat gives rise to milk fat and it seems eminent

ly possible that a proportion of the milk fatty acids, the magnitude of which is at present unknown, enters the udder as such from the blood However, the related question whether oxidative chain shortening of acyl groups acquired in this way is an important element in the fat metabolism of the udder is much more debatable in the light of more recent evidence which has directed attention to the outstanding importance of synthetic processes in this gland In this connection the experiments of Appel Bohm, Keil and Schiller (1947) with synthetic fatty acids whose molecules contain odd numbers of carbon atoms are of interest When such unnatural acids were fed to lactating sheep no fatty acids of odd number chain length below $C_{11}$ were found in the milk fat which suggests that chain shortening of natural fatty acids below the 10-carbon stage probably does not occur to a significant extent in the udder If so, then the short chain acids of milk fat must mainly arise by some process other than by degradation of long chain acids

## SYNTHESIS OF FATTY ACIDS IN THE MAMMARY GLAND

In the last few years attention has been focussed on the possibility that a large proportion of the milk fatty acids are formed by synthesis from small molecules' in the udder itself The possibility that the conversion of carbohydrate to fat can occur in the mammary gland was first suggested some eighteen years ago by Graham Houchin Peterson and Turner (1938) who studied the arterio venous differences in oxygen and carbon dioxide across the udder of the lactating goat and obtained values for the udder respiratory quotient (R Q ) which were greater than unity An R Q greater than unity is usually taken to imply the synthesis of oxygen poor substances such as fat from oxygen rich material such as carbohydrate These results were later confirmed by Reineke, Stonecipher and Turner (1941) who moreover found that fasting decreased the R Q of the goat udder below unity Since it was known from the previous work of Smith and Dastur (1938) on the lactating cow that fasting seriously depletes the milk fat of its short chain acids, Reineke *et al* deduced from their results that the short chain acids are formed in the udder from carbohydrate

In our laboratory we have shown that slices of mammary gland from lactating rats, mice guinea pigs and rabbits respire actively in the presence of glucose as sole substrate and exhibit an R Q greater than unity (Folley and French, 1949) Some of our mean values for the R Q of mammary slices from these species incubated in a medium containing glucose are shown in Table VI We interpreted these results as evidence that the mammary tissue of these species is capable of effecting net fat synthesis from carbohydrate *in vitro* The Table also

TABLE VI

*Respiratory metabolism of lactating mammary gland slices in presence of glucose* (0 3 %)

(from Folley and French 1949)

| | $-Q_{O_2}$ | R Q |
|---|---|---|
| Mouse | 16 0 | 1 94 |
| Rat | 9 3 | 1 53 |
| Guinea pig | 9 0 | 1 17 |
| Rabbit | 6 6 | 1 45 |
| Sheep | 3 9 | 0 88 |
| Goat | 4 6 | 0 86 |
| Cow | 3 5 | 0 80 |

shows that by contrast with our findings with mammary tissue from non ruminants we found that slices of lactating udder tissue from sheep goats and cows exhibited relatively little oxygen uptake in the presence of glucose alone and the R Q was below unity (Folley and French 1949 1950) This suggests that though carbohydrate might be a significant substrate for fat synthesis in the mammary gland of non ruminants it probably plays very little part in the fatty acid metabolism of the udder of ruminants This conclusion of six years ago has been subsequently confirmed in our laboratory in experiments carried out in collaboration with Dr Judith H Balmain and Dr R F Glascock in which mammary gland slices were incubated with isotopically labelled substrates including glucose labelled uniformly with $^{14}C$ Measurements

of the isotope content of the mixed fatty acids isolated from the slices after incubation for three hours in a mixture of acetate and glucose each labelled with a different isotope (acetate $^{13}C$ and $^{3}H$, glucose $^{14}C$) have enabled us to draw conclusions about the relative contributions of glucose and acetate carbon to the fatty acids synthesized by the tissue during the experiment (Balmain, Folley and Glascock, 1954) A summary of our results relating to the utilization of glucose is given in Table V 2, which shows that whereas, in the presence of a mixture of acetate and glucose the latter contributes about 60 per cent of the carbon of the fatty acids synthesized by rat mammary slices, in the case of sheep udder slices the contribution of glucose is negligible amounting to no more than about

TABLE V 2

*Incorporation of glucose carbon into the fatty acids of lactating mammary gland slices incubated with* $C^{3}H_{3}{}^{13}COONa + {}^{14}C$ *glucose*

(from Balmain Folley and Glascock, 1954)

| | Glucose carbon incorporated into mixed fatty acids of slices in 3 hr mg/100 mg | per cent carbon of fatty acids synthesized during experiment coming from glucose |
|---|---|---|
| Rat | 1 8 | 62 |
| Sheep | 0 2 | 3 |

3 per cent The conclusion that carbohydrate *is not* an important source of fatty acids synthesized by the ruminant udder does not necessarily conflict with the high R Q found for the udder of the ruminant *in vivo* for clearly the utilization of acetate for fat synthesis could give rise to a high R Q since acetate is just as much an oxygen rich' substance as carbohydrate

The incorporation of glucose carbon into the fatty acids of milk fat has been studied in the lactating mammary gland of the rabbit *in vivo* by Popják, Hunter and French (1953) They

administered glucose uniformly labelled with $^{14}C$ to lactating rabbits and isolated radioactive fatty acids from their milk Measurements of the specific radioactivity of the volatile fraction of the milk fatty acids enabled them to conclude that about 25 per cent of the carbon of the short chain fatty acids arose from glucose during the six hours following the injection These findings are not in conflict with our experiments on rat mammary gland slices

The concept that acetate is an important building unit for fatty acid synthesis in the mammary gland has arisen only in recent years Though it appears self evident today it was not so eleven years ago when the idea first arose in our laboratory In order to preserve a degree of historical perspective it may be of interest to recall the three considerations which may be regarded as having given rise to this fruitful idea The first was the demonstration by Rittenberg and Bloch (1945) and others made possible by the use of acetate labelled first with deuterium and later with $^{14}C$, that the liver will actively incorporate acetate into fatty acid chains The second was the accumulation of evidence notably by the late Sir Joseph Barcroft and his school (see Elsden and Phillipson, 1948) that in the paunch of ruminants microorganisms ferment dietary polysaccharides yielding volatile fatty acids of which the most important quantitatively appears to be acetate It is now known that much of the acetate so produced is absorbed into the blood stream giving rise to appreciable levels of acetate in the arterial blood so that acetate arising from digestion of food must be reckoned an important metabolite in the body of the ruminant Lastly there is the fact established by the chemical work of Hilditch and others that the milk fat of ruminants is distinguished even among that of herbivorous animals by a relatively high content of short chain acids This is shown in Table V 3 taken from Hilditch (1947), which gives the Reichert Meissl values for milk fats of various species The Reichert Meissl value is a measure of the steam volatile fatty acids butyric and caproic The last two of the above considerations taken together suggested to the present author and his colleagues (see Malpress 1946) that the short chain acids of ruminant milk might be formed by successive condensation of acetate molecules and might therefore be regarded as

TABLE V 3

*Short chain fatty acids ($C_4$ $C_6$) in milk fat of various species*
(from Hilditch, 1947)

| | Reichert Meissl value |
|---|---|
| *Ruminants* | |
| Cow | 33–36 |
| Buffalo | 26–34 |
| Sheep | 23–33 |
| Goat | 20–29 |
| Camel | 16 4 |
| *Non Ruminants* | |
| Rabbit | 16 1 |
| Donkey | 13 1 |
| Horse | 7 0 |
| Cat | 4 4 |
| Mouse | 2 9 |
| Man | 1 4–3 4 |
| Pig | 1 7 |
| Dog | 1 2 |

intermediate stages in the synthesis of long chain acids by similar step wise condensation

The first efforts to obtain evidence in favour of this hypothesis of the origin of the short chain acids and thus to gain insight into the mechanism of the formation of milk fat, were not encouraging As mentioned previously fasting lowers the proportion of short chain acids in the milk fat of ruminants Dr F H Malpress and I therefore felt that the fasted goat might be a suitable preparation with which to demonstrate a relationship between the acetate of the blood and the short chain acids of milk fat Accordingly, we administered sodium acetate by slow intravenous infusion to anaesthetized fasting goats but were unable to detect any resulting increase in the depleted short chain acids of the milk fat (see Malpress, 1946) Similar negative results were reported by Mann and Shaw (1947) and by McClymont (1951) in experiments in which large quantities of acetate were administered often over

prolonged periods, to fasted lactating cows either intravenously or by intraruminal infusion In view of the unequivocal evidence obtained by other techniques that the ruminant udder uses acetate for fatty acid synthesis, which will now be discussed, the failure of this approach is difficult to understand and has never been satisfactorily explained

The first positive evidence suggesting the utilization of acetate for fatty acid synthesis in the mammary gland came from respiratory measurements on mammary gland slices carried out in collaboration with the late Dr T H French in our laboratory In these experiments (Folley and French 1950), the results of which are summarized in Table V 4 we found that lactating udder slices from sheep, goats and cows

TABLE V 4

*Respiratory metabolism of lactating mammary gland slices in presence of acetate* (0 02 *M*)

(from Folley and French 1950)

| | $-Q_{O_2}$ | R Q |
|---|---|---|
| Rabbit | 6 8 | 0 92 |
| Rat | 5 3 | 0 82 |
| Sheep | 6 1 | 1 17 |
| Goat | 6 1 | 1 17 |
| Cow | 4 4 | 1 12 |

that is from ruminants respired actively with an R Q greater than unity when incubated with acetate as sole substrate and utilized considerable amounts of acetate On the other hand as the Table shows udder slices from non ruminants appeared to be more or less inert to acetate since with acetate as sole substrate the oxygen consumption was relatively low and the R Q less than unity We interpreted these findings as evidence that ruminant udder tissue will effect net fat synthesis from acetate *in vitro* From these respiratory measurements a sharp difference seemed to emerge between the metabolism *in vitro* of mammary tissue from ruminants on the one hand and non ruminants on the other, the former could apparently synthesize fat from acetate but not glucose while the latter utilized

glucose but not acetate (by itself) for lipogenesis The situation is, however, somewhat more complicated than this Bloch and Kramer (1948) found that glucose stimulates lipogenesis from acetate in rat liver slices and in our laboratory we have demonstrated a similar phenomenon in mammary tissue Thus in the presence of glucose, mammary gland slices from rats will utilize acetate the R Q in mixtures of acetate and glucose being at least as high as it is in presence of glucose alone Similarly in mixtures of acetate and glucose, sheep udder slices show a higher R Q and acetate uptake than in presence of acetate alone (Folley and French, 1950)

All the conclusions drawn from respiratory measurements, as just outlined, were later confirmed by experiments in which mammary gland slices were incubated with labelled substrates and the mean isotope content of the mixed fatty acids, isolated from the slices at the end of the incubation, determined The results of our earlier isotope experiments (Balmain Folley and Glascock 1952*a*) in which we used [*carboxy*-$^{14}$C] acetate as labelled substrate are summarized in Table V 5 The results in this Table are expressed as specific activities of the mixed fatty acids isolated from the slices as calcium

TABLE V 5

*Specific activities (counts/min/mg C) of calcium salts of mixed fatty acids isolated from lactating mammary gland slices incubated with $CH_3$ $^{14}COONa$ or $CH_3$ $^{14}COONa$ + glucose*

(results from Balmain, Folley and Glascock, 1952*a*)

| RAT | | | SHEEP | | |
|---|---|---|---|---|---|
| Experiment No | Glucose — | Glucose + | Experiment No | Glucose — | Glucose + |
| 1 | 50 | 5,951 | 1L | 3,334 | 9 533 |
| 2 | 43 | 3,805 | 1R | 3,539 | 9,911 |
| 3 | 40 | 5 886 | 2L | 2,660 | 8 975 |
| 4 | 50 | 3,532 | 2R | 2 948 | 10 249 |
| 5 | 58 | 3,215 | 3L | 4,144 | 8,068 |
| 6 | 40 | 1,069 | 3R | 4,108 | 7 441 |

salts after three hours incubation They show that rat mammary gland incorporated hardly any acetate carbon into the fatty acids when incubated with acetate as sole substrate but there was considerable incorporation in presence of glucose Udder slices from lactating sheep on the other hand incorporated considerable amounts of acetate carbon into the fatty acids when incubated with acetate alone but here again the incorporation was greatly increased when glucose was present The mechanism of the stimulatory effect of glucose upon the utilization of acetate for lipogenesis *in vitro* is not clear even yet It has been suggested that glucose provides a source of the energy which is necessary for lipogenesis to proceed but this would hardly apply to sheep mammary tissue which unlike rat mammary tissue can obtain energy from the oxidation of acetate

The question of the relative values of acetate and glucose as sources of carbon for lipogenesis in the mammary gland is of interest particularly in view of the species differences in mammary metabolism already touched upon In our subsequent experiments therefore we have studied this question by incubating mammary slices in mixtures of acetate and glucose each labelled with different isotopes (Balmain Folley and Glascock, 1954) The glucose was uniformly labelled with $^{14}C$ and the acetate was labelled in the carboxyl group with $^{13}C$ and in the methyl group with radioactive hydrogen (tritium) Our results are summarised in Table V 6 which shows the abundance of each isotope in the fatty acids isolated from the tissue These experiments have shown that rat mammary slices incorporate into the fatty acids in three hours about eight times as much glucose carbon as do sheep udder slices On the other hand sheep udder slices incorporate about six times as much of either carbon atom of acetate into the fatty acids as do rat mammary gland slices Since the mammary slices from both rat and sheep had substantially the same fat content and the abundances of the isotopes in the substrates are known, it is possible from these results to calculate the relative contributions of acetate and glucose carbon to the fatty acids newly synthesized during the experiment. These calculations, the results of which are given in Table V 7 show that in the case of rat mammary gland roughly

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(results from Balmain, Folley and Glascock, 1952a)

| RAT | | | SHEEP | | |
|---|---|---|---|---|---|
| Experiment No | Glucose — | Glucose + | Experiment No | Glucose — | Glucose + |
| 1 | 50 | 5 951 | 1L | 3,334 | 9,533 |
| 2 | 43 | 3,805 | 1R | 3,539 | 9,911 |
| 3 | 40 | 5 886 | 2L | 2 660 | 8,975 |
| 4 | 50 | 3,532 | 2R | 2,948 | 10 249 |
| 5 | 58 | 3,215 | 3L | 4,144 | 8 068 |
| 6 | 40 | 1,069 | 3R | 4 108 | 7 441 |

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equal contributions are made by acetate and glucose In the case of sheep udder slices however, the result was quite different since about 97 per cent of the carbon of the newly

TABLE V 6

*Relative incorporation of glucose and acetate carbon into the fatty acids of mammary gland slices from lactating rats and sheep the substrates were $C^3H_3$ $^{13}COONa$ + $^{14}C$ glucose*

(from Balmain, Folley and Glascock, 1954)

| Species | | | | | | | Mean | Ratio a/b |
|---|---|---|---|---|---|---|---|---|
| | *$^{14}C$ (glucose carbon) counts/min/mg C* | | | | | | | |
| Rat | 307 | 381 | 124 | 245 | 110 | 170 | 223(a) | 7 9 |
| Sheep | 38 4 | 17 4 | 22 5 | 24 7 | 35 8 | 31 6 | 28(b) | |
| | *$^3H$ (acetate methyl carbon) counts/min/mg combustion water* | | | | | | | |
| Rat | 84 5 | 94 8 | 41 7 | 109 5 | 43 9 | 71 2 | 73 3(b) | 6 3 |
| Sheep | 421 | 405 | 501 | 502 | 451 | 502 | 464(a) | |
| | *$^{13}C$ (acetate carboxyl carbon) atoms per cent excess* | | | | | | | |
| Rat | 0 07 | 0 05 | – | 0 06 | – | 0 04 | 0 055(b) | 5 9 |
| Sheep | 0 252 | 0 249 | 0 373 | 0 378 | 0 370 | 0 320 | 0 324 (a) | |

TABLE V 7

*Incorporation of substrate carbon into the fatty acids of lactating mammary gland slices utilizing acetate and glucose*

(from Balmain Folley and Glascock, 1954)

| | Acetate ($^{13}C$) | Glucose ($^{14}C$) |
|---|---|---|
| Rat | 1 1 | 1 8 |
| Sheep | 6 5 | 0 2 |

The results are expressed as mg of substrate carbon incorporated into 100 mg fatty acid carbon in 3 hr

formed fatty acids came from acetate It thus appears that ruminant mammary tissue differs from rat mammary tissue in that it can only very slowly degrade glucose to the active two carbon unit, acetyl coenzyme A, now believed to be the form in which acetate enters the pathway of lipogenesis or else the Krebs cycle for oxidation This conclusion has interesting implications, for Duncombe and Glascock (1953) in our Institute have recently shown that sheep mammary gland slices incubated with glucose labelled uniformly with $^{14}C$ can produce radioactive carbon dioxide which means that sheep udder tissue can oxidize glucose even when present in the incubation medium as sole substrate Mammary tissue may therefore be able to oxidize glucose by some pathway not involving the glycolytic route and it seems likely that the direct glucose oxidation cycle (see Dickens, 1953 for review) operates in the mammary gland Evidence in favour of this suggestion for the rat mammary gland has recently been reported by Glock and McLean (1954) and Abraham Hirsch and Chaikoff (1954)

The most conclusive evidence of the utilization of acetate for the synthesis of milk fat in the ruminant udder comes from experiments carried out on lactating animals in vivo Popják and Beeckmans (1950) administered deuterium or [*carboxy*-$^{14}C$] acetate to pregnant rabbits and showed that fat isolated from the mammary glands of these rabbits was in each case isotopically labelled Much of the fat present in the mammary gland in late pregnancy may well be depot fat or structural lipids but the fact that in collaboration with Dr Popjak we were able to isolate short chain fatty acids from the mammary gland fat of his rabbits shows that some synthesis of true milk fat proceeds even before parturition (Popjak, Folley and French 1950) Assays for $^{14}C$ were carried out on various fractions of these acids with results that are shown in Table V 8 It will be seen that the short chain acids were highly radioactive by comparison with the long chain acids and moreover they were much more radioactive than the mixed fatty acids isolated from the liver fat These results permitted three deductions first that the short chain acids could not have arisen directly from the liver fatty acids and by implication from fatty acids circulating in the blood, secondly, that

TABLE V 8

*Specific activities of the glyceride fatty acids from the mammae and livers of rabbits killed at the 28th day of pregnancy after treatment with $CH_3{}^{14}COONa$*

(from Popjak, Folley and French, 1949)

| Fatty acid fraction | Specific activity of fatty acids $\mu c \times 10^{-14}$/mg C | | |
|---|---|---|---|
| | Rabbit no | | |
| | 10 | 13 | 15 |
| Volatile water-soluble | 1 98 | 0 73 | 18 45 |
| Volatile, water insoluble | 1 48 | 0 72 | 13 60 |
| Non volatile | 0 12 | 0 07 | 0 99 |
| Liver fatty acids | 0 146 | 0 183 | 0 58 |

they could not have been formed by the degradation of long chain acids of the mammary gland fat and thirdly, that they probably arose by synthesis from acetate in the mammary gland itself

In view of these interesting results it was decided to extend this work to a detailed study of the secretion of true milk fat in the lactating ruminant Accordingly 5 mc [*carboxy*-$^{14}$C] acetate were injected intravenously into a lactating goat and milk samples taken at frequent intervals after the injection (Popjak, French and Folley, 1951) Glyceride fatty acids were isolated from the milk and separated into four fractions steam volatile acids, soluble in water, steam volatile acids, insoluble in water non volatile saturated acids, non volatile unsaturated acids The specific activity time curves for all four fractions are shown in Figure V 1 It will be seen that all the curves showed maxima at 3–4 hr after the injection, but the mean specific activities of the two volatile fractions at their maxima were several times greater than those of the two non volatile fractions Moreover the specific activities of the fatty acids of the plasma fat were so much lower at all periods of the experiment than those of any fraction of the

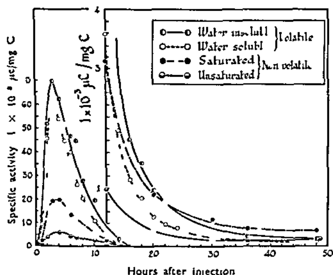



Fig V 1 Specific activity time curves of glyceride fatty acid fractions isolated from the milk of a goat after the intravenous injection of 5 mc of [*carboxy*-$^{14}$C] acetate (*from Popják French and Folley 1951*)

milk fatty acids that they cannot be shown in the same diagram These results showed that the synthesis of fatty acids from acetate is of great importance in the fat metabolism of the goat udder and moreover that the short chain acids must arise by synthesis from 2 carbon units within the udder rather than by oxidative degradation of long chain acids as postulated by the theory of Hilditch Later Dr Popjak and his colleagues together with the late Dr T H French of our laboratory in continuation of this joint project isolated individual fatty acids from these crude fractions and by determination of the specific radioactivities of these pure fatty acids and of individual carbon atoms obtained from the butyric caproic and caprylic acids by chemical degradation showed that chain elongation must take place by successive addition of two-carbon units to the carboxyl end of the fatty acid chain (Popjak French Hunter and Martin 1951) This provided conclusive evidence in favour of our previous contention (see Folley and French 1948 Folley, 1949) that the short chain acids of milk

fat are intermediates in the synthesis of the long chain acids from small molecules Another important point emerged from this chemical work of Popjak *et al* Figure V 2, for which I am indebted to Dr G Popjak, shows the mean specific activities of all the saturated fatty acids, present in these milk

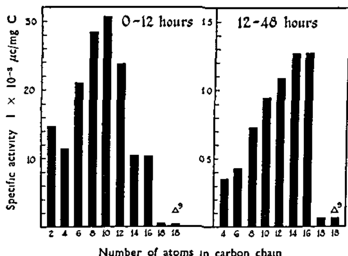



Fig V 2 Specific activity of milk fatty acids after injection of 5 mc of [*carboxy* [1] C] acetate into a lactating goat *(figure constructed from the results of Popják French Hunter and Martin 1951)*

samples, from butyric to stearic together with that of the oleic acid It will be noted that there is a sharp drop in specific activity between palmitic acid on the one hand and oleic and stearic acids on the other, which suggests that the $C_{18}$ acids of milk fat, and probably all the higher ones, must be mainly formed by some mechanism other than by synthesis in the udder It seems likely that they arise chiefly from the blood glycerides as suggested by the arterio venous differences already mentioned Finally, the specific activities of the individual carbon atoms of the caproic acid suggested that the radioactive butyrate formed in the udder of the experimental goat by condensation of two acetate molecules was continually diluted by a non isotopic four carbon compound arising from

the blood The blood fat of ruminants contains relatively little butyric acid but there are considerable amounts of $\beta$ hydroxy butyrate present It thus seemed possible that the four carbon compound which appears to serve as an additional substrate for lipogenesis in the goat might be $\beta$ hydroxybutyrate This would agree with the fact that Shaw and Knodt (1941) have observed arterio venous differences in $\beta$ hydroxybutyrate concentration amounting to about 2 5 mg/100 ml across the lactating bovine udder However, Kleiber Black Brown, Luick Baxter and Tolbert (1954) who injected butyrate labelled with $^{14}C$ into lactating cows found that butyrate carbon entered the milk fatty acids to a much smaller extent than acetate carbon About 70 % of the butyrate carbon entering the organic milk constituents was found in lactose and casein The utilization of acetate labelled with $^{14}C$ for fatty acid synthesis in the lactating mammary gland *in vivo* has also been observed by Kleiber, Smith, Black Brown and Tolbert (1952) in the cow and by Popjak, Hunter and French (1953) in the rabbit

In summary then we can say that in ruminants a proportion of the fatty acids of the milk glycerides is synthesized in the udder from small molecules the precursors being mainly acetate and perhaps $\beta$ hydroxybutyrate It is probable that most of the fatty acids up to and including palmitic acid are formed in this way while the oleic, stearic and higher acids probably arise mainly from the blood glycerides In the mammary gland of non ruminants, as exemplified by the rat and rabbit a similar mechanism almost certainly obtains save that considerable quantities of glucose carbon are also used for lipogenesis Information on the proportion of the long chain acids of ruminant milk fat arising from the blood glycerides would be of great interest It is being sought in this Institute at the time of writing by Duncombe and Glascock (1954) who are feeding tritio stearic acid prepared by the tritiation of elaidic acid to lactating goats and cows Their latest preliminary experiments (unpublished) indicate that some 20—30 % of the long chain acids originate from the blood fatty acids

An interesting development in the study of the fatty acid metabolism of mammary tissue is the demonstration by Popják and Tietz (1954) and later in our laboratory by Terner (1954)

that cell free homogenates of mammary gland will synthesize fatty acids from acetate The former workers have now prepared a soluble enzyme system from mammary tissue capable of effecting this synthesis and have shown that it is coenzyme A dependant (Popják and Tietz, 1955, Tietz and Popják, 1955)

THE ORIGIN OF THE GLYCEROL OF THE MILK GLYCERIDES

So far we have only discussed the origin of the fatty acids of the milk fat The mammary gland, however, secretes mainly glycerides so that the source of the glycerol must also be considered Though the lactating udder absorbs glycerides from the blood the fact that it actively synthesizes fatty acids suggests that it does not acquire all the glycerol it needs in the form of glycerides Popják Hunter and French (1953) in their experiments in which $^{14}C$ glucose was administered to lactating rabbits found that the glycerol of the milk glycerides was strongly labelled, its specific activity being about equal to that of the milk lactose They concluded that between 65 and 95 per cent of the glyceride glycerol was formed from glucose in the mammary gland during the six hours after the injection The experiment of Popják, French and Folley (1951), mentioned above, in which 5 mc of $^{14}C$ labelled acetate were injected into a lactating goat provided further evidence upon this question since the milk lactose and glyceride glycerol became labelled Figure V 3 shows the specific activity time curves for lactose and glyceride glycerol isolated from the milk of this goat (Popják, Glascock and Folley, 1952) It will be seen that the lactose curve rises to a maximum and then declines exponentially the glycerol curve remains below the lactose curve until it in turn reaches its maximum at which point it crosses the lactose curve This is precisely the relationship which according to Zilversmit, Entenman and Fishler (1943), should hold between the curves for a reaction product, in this case the glycerol curve, and its immediate precursor, in this case the lactose curve It is not suggested, however, that lactose is the actual precursor of glycerol it is much more likely that the immediate precursor of glycerol is glucose and that under these conditions lactose and glucose are in rapid equilibrium so that the specific activities of the two sugars are

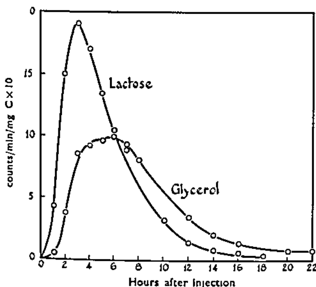



Fig V 3 Specific activity time curves for lactose and glycerol isolated from the milk of a goat which had been injected intravenously with 5 mc of [*carboxy* $^{14}$C] acetate (*from Popják Glascock and Folley 1952*)

the same at any moment The results shown in the Figure suggest strongly that the conversion of glucose to glycerol takes place not in the liver but in the mammary gland itself

## HORMONAL CONTROL OF LIPOGENESIS IN THE MAMMARY GLAND

In conclusion let us discuss the hormonal control of lipo genesis in the mammary gland We will begin with insulin because so far more work has been done on the role of insulin in fatty acid synthesis than on that of any other hormone Experiments on the living animal by Drury (1940) Stetten and Boxer (1944) and others suggested that an important action of insulin is to promote the formation of fat from carbohydrate It follows that the hypoglycaemic action of insulin might partly be due to its action in directing carbohy drate along this pathway of metabolism Bloch and Kramer (1948) were the first to demonstrate a stimulatory effect of

insulin on lipogenesis *in vitro* They found that rat liver slices incubated with pyruvate and $^{14}C$ labelled acetate incorporated more acetate carbon into the fatty acids in the presence of insulin than in its absence Though these authors could not confirm these results in a different strain of rat, similar and more convincing effects of insulin on lipogenesis in rat liver slices were later reported by Brady and Gurin (1950) As our group has more than once pointed out, the outstanding capabilities of mammary tissue for effecting net fat synthesis *in vitro* make it an eminently suitable medium for studying lipogenesis, and it is perhaps not surprising that we have been able to demonstrate particularly clear cut even dramatic, stimulatory effects of insulin on lipogenesis in mammary gland slices

In all our work we have used insulin free from the hyper glycaemic factor which is present in many insulin preparations and might confuse the issue We have found that if mammary gland slices from rats in full lactation are incubated with a mixture of acetate and glucose, addition of insulin to the medium markedly increases the R Q and also the uptake of both acetate and glucose Some of our earlier results (Balmain, French and Folley, 1950) are shown in Table V 9 With glucose alone as substrate similar but somewhat smaller effects were observed but insulin had no ameliorating effect on the

TABLE V 9

*Effect of insulin added* in vitro *on the utilization of acetate and glucose by lactating mammary gland slices*

(from Balmain, French and Folley 1950)

| Number of rats | Addition | Glucose | | | Glucose + acetate | | |
|---|---|---|---|---|---|---|---|
| | | $-Q_{O_2}$ | $Q_{acid}$ | R Q | $-Q_{O_2}$ | $Q_{acid}$ | R Q |
| 10 | Insulin (1·1 u/ml) | 10·4 | 1·7 | 1·80 | 13·4 | −4·6 | 2·03 |
| | No insulin | 9·2 | 1·7 | 1·57 | 10·5 | −2·1 | 1·53 |
| P | | n s | n s | $<0·001$ | $<0·02$ | $<0·001$ | $<0·001$ |

indifference of rat mammary slices to acetate (Balmain and Folley 1951) The lowest concentration of insulin to give detectable effects was found to be in the neighbourhood of 2 mu/ml. (Balmain, Cox Folley and McNaught 1954) We interpreted these changes as indicating that insulin stimulates lipogenesis from small molecules in rat mammary gland slices That this interpretation was justified was subsequently shown in experiments in which mammary gland slices were incubated with labelled substrates Table V 10 shows results

TABLE V 10

*Effect of insulin, added* in vitro *on lipogenesis in rat mammary gland slices incubated with $C^3H_3{}^{13}COONa$ + $^{14}C$-glucose*

(from Balmain, Folley and Glascock 1954)

| | Isotope content of calcium salts of mixed fatty acids after 3 hr | | |
|---|---|---|---|
| | $^{14}C$ counts/min/mg *C* | $^3H$ counts/min/mg combustion water | $^{13}C$ atoms per cent excess |
| Control | 381 | 95 | 0 05 |
| Insulin (1 0 i u/ml) | 805 | 202 | 0 14 |

from an experiment in which mammary gland slices from lactating rats were incubated with $^{14}C$-glucose together with [*carboxy* $^{13}C$-*Me*$^3H$] (Balmain Folley and Glascock 1954) The results show that insulin increases the incorporation of glucose carbon and also of both carbons of acetate into the fatty acids of the slices

In comparing these results with those obtained with mammary tissue from lactating ewes interesting species differences are again apparent for we have never found any stimulatory effects of insulin added *in vitro* upon lipogenesis in sheep mammary tissue either by respiratory methods (Balmain and Folley 1951) or by the use of isotopically labelled substrates (Balmain Folley and Glascock 1954) Results obtained with labelled substrates are shown in Table V 11 Udder slices from lactating ewes were incubated with acetate and glucose

TABLE V 11

*Effect of insulin, added* in vitro, *on lipogenesis in sheep udder slices incubated with $C^3H_3{}^{13}COONa + {}^{14}C$ glucose*

(from Balmain Folley and Glascock, 1954)

| | Isotope content of calcium salts of mixed fatty acids after 3 hr | | |
|---|---|---|---|
| | $^{14}C$ counts/min/mg C | $^{3}H$ counts/min/mg combustion water | $^{13}C$ atoms per cent excess |
| Control | 22 5 | 501* | 0 37 |
| Insulin (1 0 1 u /ml ) | 27 6 | 485* | 0 33 |

* These values are one half of those given for sheep udder half S5L in Table 7 of the original paper (Balmain *et al* 1954) All the values of the series to which they belong were halved for inclusion in Table 1 of the original paper (reproduced in this book as Table V 6) so as to make them comparable with the values for the rat

labelled with different isotopes as before The results show that in no case did insulin increase the abundance of any of the isotopes in the fatty acids It will be recalled that we found that whereas under these conditions rat mammary tissue utilizes acetate and glucose to approximately equal extents for fatty acid synthesis, sheep udder slices use acetate almost exclusively for this purpose and incorporate practically no glucose It therefore seems reasonable to suggest that the effect of insulin on lipogenesis is bound up with its effect on some metabolic system which is present in rat mammary tissue but absent from the mammary tissue of the ewe In view of what was said earlier it would seem that this must be the system which produces acetyl coenzyme A from glucose Our results in general are in harmony with the view that the effect of insulin on lipogenesis is exerted at some point in the breakdown of glucose to acetyl coenzyme A rather than on the utilization of acetyl coenzyme A for fatty acid synthesis

It is well known that the 11-oxygenated steroids secreted by the adrenal cortex exert an anti insulin effect in certain respects The possibility that the adrenal cortex might play a role

in the control of lipogenesis must therefore also be considered, since one might expect that adrenal steroids would exert an opposite effect on lipogenesis to that of insulin Evidence of such antagonistic effects was provided by Brady Lukens and Gurin (1951) who showed that pre treatment of rats with cortisone decreased the incorporation of labelled acetate carbon into the fatty acids of slices of their livers In our laboratory we have studied the effects of various adrenal steroids added *in vitro*, on lipogenesis in lactating mammary gland slices We have found that cortisone added to the medium inhibits the incorporation of both acetate and glucose carbon into the fatty acids of rat mammary gland slices in cubated with [$Me\ {}^3H$] acetate and $^{14}C$ glucose When both hormones were present cortisone partially inhibited the stimu latory effect of insulin on lipogenesis (Balmain Folley and Glascock 1952*b*) Results of a typical experiment illustrating these facts are shown in Table V 12 In subsequent experi ments (McNaught, Glascock Balmain and Folley 1955)

TABLE V 12

*Effect of cortisone and insulin on the incorporation of* $^3H$ *and* $^{14}C$ *into the mixed fatty acids of lactating rat mammary gland slices incubated with* $C^3H_3COONa + {}^{14}C$ *glucose*

(from Balmain Folley and Glascock, 1952*b*)

| Addition | $^{14}C$ in the fatty acids | | $^3H$ in the fatty acids | |
|---|---|---|---|---|
| | counts/min /mg C | per cent of control | counts/min /mg $H_2O$ | per cent of control |
| None | 365 | 100 | 283 | 100 |
| Cortisone | 254 | 70 | 77 | 27 |
| Insulin (0 01 i u /ml ) | 795 | 218 | 430 | 152 |
| Insulin (0 01 i u /ml ) +cortisone (100 $\mu$g/ml ) | 383 | 105 | 118 | 42 |

similar inhibitory effects on lipogenesis have also been obtained with deoxycorticosterone and corticosterone though these steroids were more active than cortisone Different results

were however given by cortisol (hydrocortisone) This steroid at the same concentration, 100μg/ml, had no inhibitory effect on lipogenesis, nor did it interfere with the stimulatory action of insulin Since there is some reason to believe that cortisol may be the natural glucocorticoid secreted by the adrenal cortex of the rat, the fact that we have found its action on lipogenesis *in vitro* to be different from that of two other glucocorticoids, cortisone and corticosterone, is of considerable interest Sheep mammary gland slices once again have been found to behave differently from those of the rat, this time in respect of the effects of adrenal corticoids on lipogenesis *in vitro* as measured by the rate of incorporation of labelled acetate carbon into fatty acids (McNaught *et al* 1955) In sheep udder slices cortisone and cortisol at a concentration of 100 μg/ml have been found to exert no regular effects on lipogenesis either way, while corticosterone and deoxycorticosterone in similar concentrations strongly inhibit lipogenesis as they do in rat mammary slices These results are difficult to interpret at the present time particularly since the concentrations used are some 1000 times greater than those believed to occur in the blood stream, so that it is not yet possible to draw firm conclusions about the role of the adrenal cortex in the control of lipogenesis Clearly further work is needed upon this aspect of the subject

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# CHAPTER VI

## RECENT STUDIES OF THE BIOSYNTHESIS OF LACTOSE AND MILK PROTEINS

### THE ORIGIN OF THE LACTOSE OF MILK

Lactose, the carbohydrate of milk is a disaccharide glucose 4 $\beta$ galactoside(4–O–$\beta$–D–galactopyranosyl D glucopyranose), the structural formula of which ($\alpha$ form) is shown in Figure VI 1 Lactose is normally found nowhere in the body but in the mammary gland Since it is not present in blood except

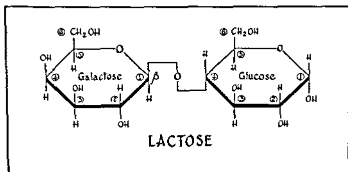



Fig VI 1

when for one reason or another suckling or milking have ceased so that the gland is temporarily engorged with milk lactose must be synthesized in the gland itself In view of its intrinsic interest the problem of how lactose is formed has stimulated the imagination of many investigators but no universally acceptable and final solution has yet been reached Recent progress however encourages the hope that the resolution of this seemingly simple yet baffling enigma will soon be achieved

There are two main possibilities to consider Lactose may be synthesized from small molecules, that is two or three carbon units, or it may be made from preformed hexose units taken from the blood It is, of course, conceivable or perhaps even probable that the mammary gland may make lactose partly from preformed hexose molecules and partly from smaller molecules With regard to the first possibility there is no conclusive evidence that lactose synthesis from small molecules cannot occur to some extent in the lactating gland Though arterio venous studies on cows (Shaw, 1946) have provided no evidence that the udder utilizes blood lactic acid or pyruvate which are the "small molecular" precursors which come first to mind, they have consistently revealed an uptake of amino acids (Graham, 1937, Shaw and Petersen 1938, Reineke Peterson Houchin and Turner, 1939), some fraction of which, provided the mammary gland can carry out the necessary deamination, could provide building units for lactose synthesis The suggestion that blood amino acids might serve as substrates for lactose synthesis was made nineteen years ago by Graham (1937) However the most probable role for amino acids in the mammary gland is to serve as material for the synthesis of milk proteins and since, as we shall see later, it has been contended that the amino acid uptake is insufficient for this purpose, it seems unlikely that there would be any to spare for lactose synthesis Furthermore, as was pointed out in Chapter III, there is no good evidence for the occurrence of gluconeogenesis in the mammary gland of any species other than the rat and mouse The possibilities are not exhausted, however, since arterio venous studies on the bovine udder have revealed uptakes of acetate (McClymont 1951) and β hydroxybutyrate (Shaw and Knodt, 1941) both of which could conceivably be built into the carbon skeleton of lactose Indeed a perfusion experiment carried out by our group in collaboration with Dr G Popják of London and Professors G Peeters and L Massart of Gent has shown that the isolated bovine udder can incorporate acetate carbon into lactose (Cowie, Duncombe, Folley, French, Glascock, Massart, Peeters and Popják, 1951) We added [*carboxy*-$^{14}$C] acetate to the blood perfusing a bovine udder-half and were able to isolate radioactive lactose from the milk

This suggests that the udder can synthesize some lactose from small molecules but does not prove that a net synthesis of carbohydrate from acetate is possible in the isolated udder Apparently the udder incorporates acetate carbon into lactose by some mechanism other than oxidation followed by fixation of the resulting carbon dioxide since lactose secreted by the contralateral udder half, which was simultaneously perfused with radioactive bicarbonate had no appreciable radioactivity More recent work with the perfused bovine udder carried out by Dimant Smith and Lardy (1953) strongly suggests that lactose is formed in the udder mainly from preformed hexoses probably glucose They perfused a bovine udder with blood containing 1 $^{14}C$ glucose and found that in the glucose and galactose obtained by hydrolysis of the milk lactose the radioactivity was in each case mainly confined to the $C_{(1)}$ position Since this experiment was carried out on an isolated udder the interpretation of the results was not complicated by redistribution of the label resulting from reactions in other tissues such as the liver

I venture tentatively to conclude that the experimental evidence available at the present time favours the view that the mammary gland builds up lactose mainly from preformed hexose units rather than from two or three carbon units If further work should prove this to be the case, there are two principal possibilities to consider in this connection also These concern the origin of the galactose half of the lactose molecule

On the one hand we must enquire whether the mammary gland uses preformed galactose which it obtains from the blood and on the other we have to consider the alternative possibility that blood glucose is the sole precursor of lactose, the galactose half of the lactose molecule being formed by rearrangement of the glucose molecule in the mammary gland

The arterio venous studies of Reineke, Williamson and Turner (1941) on lactating goats have indicated that there is an appreciable arterio venous difference in plasma glyco protein across the lactating udder Now there is some evidence that the protein bound carbohydrate of the glycoprotein fraction of the blood plasma proteins consists apart from a glucosamine component of an equimolecular complex of

mannose and galactose (Friedmann, 1949), so that there is a strong possibility that the udder may acquire galactose from the blood We have, however, no quantitative information about whether the amount of galactose taken up by the gland in the form of protein bound carbohydrate is sufficient to account for the galactose moiety of the milk lactose but *a priori* it seems rather unlikely that this can be so A few years ago, we found in our laboratory that mannose was the only carbohydrate besides glucose of a large number tried, which increased above endogenous values the oxygen consumption and R Q of mammary gland slices from lactating rats (Folley and French 1949) This suggested that glucose and mannose are interconvertible in the mammary gland from which it would follow that mannose could be used for any purpose for which the gland uses glucose This in turn led us to speculate upon whether some proportion at least of the milk lactose might not be formed by molecular rearrangement in the mammary gland of the mannose galactose complex present in the plasma glycoprotein Malpress (1951), however later reported that guinea pig mammary slices which would synthesize lactose from glucose *in vitro* would not form lactose in presence of mannose and so his results do not support this suggestion

As regards the alternative point of view it can be said at once that there is a considerable amount of evidence that the mammary gland can synthesize lactose from glucose alone and if this is so there seems to be no need to postulate any other precursor for lactose than the glucose of the blood The idea that the blood glucose is the precursor of the milk lactose is a comparatively old one which in more recent times has found support in the fact that in numerous studies marked arterio venous differences in blood glucose across the udders of lactating goats or cows have invariably been observed More over, it can be said that in general, procedures which lower the blood sugar tend to decrease the milk lactose concentration while procedures causing hyperglycaemia have the opposite effect (see Folley, 1940 for review) The best evidence in its favour, however, comes from experiments on the synthesis of lactose by mammary gland slices which were first successfully carried out by Grant (1935) For the detection and estimation of lactose Grant devised a differential fermentation method

involving the use of yeasts adapted to ferment lactose and galactose and if we can accept Grant's method as specific for lactose, we must conclude that he was able to demonstrate a marked synthesis of lactose by slices of guinea pig mammary gland incubated with glucose as sole substrate Not only did Grant thus show that guinea pig mammary gland could form the galactose moiety of lactose from glucose but he obtained additional evidence that the provision of preformed galactose is not necessary, for he further showed (Grant 1936) that addition of galactose to the medium did not increase the amount of lactose synthesized from glucose Grant's results perhaps, should not be regarded as conclusive proof that preformed galactose is not essential since galactose might be stored in the tissue in the form of a polysaccharide In this connection it may be noted that Caputto and Trucco (1952) reported the presence of a polysaccharide containing glucose and galactose in mammary gland extracts Later they identified one of the carbohydrate complexes detected by them in mammary gland extracts as *neuramin lactose* a compound of lactose and neuraminic acid (Trucco and Caputto 1954) An essentially similar compound has also been identified in mammary tissue by Heyworth and Bacon (1954) A tetrose of the structure glucose galactose N acetylglucosamine galactose has been isolated from human milk by Kuhn Gauhe and Baer (1954) Whether either of these substances is implicated in lactose biosynthesis remains to be seen the possibility that a poly saccharide containing galactose is concerned in lactose forma tion will be referred to again later on Six years ago Malpress and Morrison (1950) demonstrated the synthesis of lactose from glucose by slices not only of guinea pig mammary gland, but also (see Malpress 1951) of lactating goat udder Their results are perhaps more conclusive than those of Grant because they used a rather specific colorimetric method for the determination of lactose Finally Reithel Horowitz Davidson and Kittinger (1952) have succeeded, by the use of $^{14}C$ glucose in demonstrating for the first time the synthesis of lactose from glucose in mammary gland homogenates By means of Reithel's technique it should be possible to make further progress in the study of intermediates in lactose synthesis In fact, his later work (Kittinger and Reithel 1953)

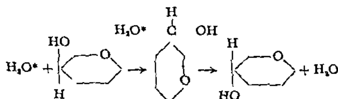
suggests that a soluble protein extracted from guinea pig mammae will catalyze a trans glycosidation reaction, involving glycogen or starch which yields lactose

Some further reasons for the belief that glucose is the main if not the sole precursor of both halves of the lactose molecule may also be mentioned French Popjak and Malpress (1952) fed $^{14}C$ starch to lactating rabbits and isolated lactose from their milk They found that the mean specific activity of the whole lactose molecule and of the glucose and galactose obtained therefrom by acid hydrolysis were all sensibly the same This led them to infer that both halves of the galactose molecule are formed equally from the same source, namely, glucose Their results suggest that if other precursors are involved to any appreciable extent they must first be converted to glucose A similar conclusion follows from the work of Dimant Smith and Lardy (1953) on the perfusion of the bovine udder with radioactive glucose already mentioned, for these workers also found that the glucose and galactose isolated from the lactose formed during the perfusion had equal specific activities On the other hand Barry (1952*a*) who injected radioactive glucose into a lactating goat, found that the specific radioactivity of the galactose isolated from the milk lactose was measurably less than that of the corresponding glucose

The next question to discuss is the mechanism of the conversion of glucose to galactose These two hexoses differ only in the steric configuration of the groups attached to $C_{(4)}$, so that all that is necessary to transform glucose to galactose is a Walden inversion at this carbon atom Nevertheless this seemingly simple conversion is extremely difficult to effect by chemical means in the laboratory and has been reported only once Some light has been thrown on this problem by studies of the converse process the conversion of galactose to glucose in adapted yeasts and also in the mammalian liver Leloir and his collaborators (Trucco Caputto Leloir and Mittelman 1948, Caputto, Leloir, Trucco Cardini and Paladini, 1949, Caputto, Leloir, Cardini and Paladini, 1950) in a series of brilliant investigations have described an enzyme system present in a yeast, *Saccharomyces fragilis*, adapted to ferment lactose, which will effect this conversion The first step is the

phosphorylation of galactose at $C_{(1)}$ by an enzyme, galacto kinase which catalyzes the transfer of phosphate from ATP The α galactose 1 phosphate formed is then transformed to α glucose 1 phosphate by an enzyme called galactowaldenase This enzyme requires a coenzyme which the Leloir group has identified as uridine diphosphate glucose, a substance which can be regarded as glucose 1 phosphate combined with uridine 5 phosphate through a pyrophosphate linkage The relevance of this work to the lactose problem is obvious *Should the various steps in this enzymatic* conversion prove to be reversible and moreover if the enzyme system were found in mammary tissue then we would have a feasible route from glucose to galactose It is therefore, of considerable interest that Caputto and Trucco (1952) have reported the detection of galactowaldenase and its coenzyme in mammary tissue and that the presence of the coenzyme in the mammary gland has been confirmed by Rutter and Hansen (1953) These interesting findings may mean that the mammary gland possesses the enzyme system necessary to effect the Walden inversion of glucose at $C_{(4)}$

Results obtained by Topper and Stetten (1951) on the conversion of galactose to glucose in the rat liver may also be relevant to the problem we are discussing H O L Fischer (1944–5) had suggested that the cyclic hexitol L inositol might be an intermediate in the interconversion of galactose and glucose To test this possibility Topper and Stetten administered 1 $^{14}C$ galactose to rats and isolated labelled glycogen from their livers Chemical degradation of the glucose obtained *by hydrolysis of this glycogen showed that the radioactivity* resided almost exclusively in $C_{(1)}$ which excluded L-inositol as an intermediate since it can be shown on theoretical grounds that this route would yield glucose labelled at $C_{(4)}$ Though the results do not entirely exclude the possibility that an *isomer of L inositol muco inositol is an intermediate* Topper and Stetten (1951) interpret them as favouring the view that the conversion of galactose to glucose in the liver proceeds by direct epimerization at $C_{(4)}$ Koshland (1954) has discussed this epimerization in terms of a single displacement mechanism which might be confirmed by experiments with $^{18}O$ labelled water as follows




The possibility was suggested by Malpress (1951), that the conversion of glucose to galactose might proceed through the cleavage of the glucose molecule to triose fragments which undergo an aldol condensation in such a way as to give the required *cis* configuration to the groups attached to $C_{(3)}$ and $C_{(4)}$. The localization in $C_{(1)}$ of the radioactivity of the galactose obtained from lactose secreted by the bovine udder which Dimant, Smith and Lardy (1953) perfused with 1 $^{14}C$ glucose, is consistent with this hypothesis if it be assumed that interconversion of aldo and keto trioses is prevented in some way for example if the first three carbon atoms of glucose remained attached to a coenzyme If there were a single three carbon intermediate there would be some redistribution of the radioactive carbon among other carbon atoms of the galactose molecule However, the fact that glycogen when synthesized in the body from galactose in presence of $D_2O$ contains no more deuterium than when glucose is the substrate (Stetten and Klein 1946) may be cited as evidence against an aldol type of cleavage

On the whole, the evidence thus far available seems to favour the suggestion that galactose is formed from glucose in the mammary gland mainly by direct epimerization at $C_{(4)}$, though the possibility that the conversion involves the cleavage of the hexose molecule followed by recombination, as suggested by Malpress, is not entirely excluded The detection in mammary tissue by Caputto and Trucco (1952), of the enzyme system, well characterized in certain galactose adapted yeasts, which can convert galactose 1 phosphate to glucose 1 phosphate, appears to provide the most suggestive circumstantial evidence yet available that this mechanism may play an important role in the biosynthesis of lactose More positive evidence will be needed however, before this conclusion can be accepted, and the work of Leloir (1951) suggests a possible way in which further evidence might be sought Leloir has

shown that an enzyme from *Saccharomyces fragilis*, presumably galactowaldenase, will reversibly transform uridine diphosphate glucose (UDPG) into uridine diphosphate galactose (UDPGal) so that an equilibrium is reached The reactions were formulated by Leloir as follows



$$\text{UDPG} + \text{Gal 1 P} \rightleftarrows \text{UDPGal} + \text{G 1 P} \quad (1)$$



$$\text{UDPGal} \rightleftarrows \text{UDPG} \quad (2)$$

Evidence in favour of this mechanism has since been provided by Trucco (1954) who showed that $^{14}C$ is incorporated into UDPG when this compound together with $^{14}C$ glucose 1 phosphate is incubated in presence of *Saccharomyces fragilis* extract The reversibility of the transformation of galactose 1 phosphate to glucose 1 phosphate by extracts of *Lactobacillus bulgaricus* has been shown by Hansen and Craine (1954) while other biological reactions which result in the formation of uridine diphosphate galactose have been studied by Kalckar Braganca, and Munch Petersen (1953) The findings of Leloir suggest that it would be interesting to carry out experiments to find out if mammary gland extracts or homogenates will effect the interconversion of uridine diphosphate glucose and uridine diphosphate galactose If such an interconversion could be demonstrated in mammary gland preparations and if further it could be shown that mammary gland extracts contain an enzyme which will split off galactose or galactose phosphate from uridine diphosphate galactose the evidence for the participation of the galactowaldenase system in the biosynthesis of lactose would be strengthened Here would seem to be a clear indication of a direction along which further investigations might be fruitful

Even if it can be shown experimentally that the mammary gland is capable of forming galactose or more probably galactose 1 phosphate by simple epimerization of glucose 1 phosphate at $C_{(4)}$ or by some other mechanism there remains the problem of how the condensation of the two hexoses is effected Some years ago the writer suggested (Folley 1949) that this condensation might be brought about by a transglycosidation reaction analogous to that demonstrated by Hassid Doudoroff and Barker (1944) for the formation of sucrose in bacteria and which Doudoroff (1945) suggested

might apply to sucrose formation in plants These authors have demonstrated the ability of a bacterial phosphorylase to catalyse the condensation of glucose 1 phosphate with fructose to give sucrose It has since been shown that sucrose phosphory lase will catalyse trans glycosidation reactions which do not involve phosphate However it must be pointed out that an analogous mechanism for the synthesis of lactose in the mam mary gland would require that mammary tissue contains a phosphoryla e specific for $\beta$ galactose 1 phosphate, that is an enzyme capable of catalysing the formation of the $\beta$ galacto sidic linkage Moreover since the galactowaldenase system appears to be concerned with the metabolism of $\alpha$ galacto e 1-phosphate in order to complete the theoretical cheme we are di cussing we may have to postulate the existence in the mam mary gland of a mutase of a type hitherto unknown, namely one capable of converting an $\alpha$ glycosidic linkage into the $\beta$ con figuration Figure VI 2, which shows this theoretical scheme

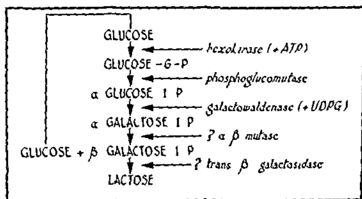



Fig VI 2

for the biosynthesis of lactose illustrates the points just dis cussed and further shows that the energy necessary for the formation of the glycosidic bond of lactose would come from ATP It must be emphasized that, however attractive this hypothesis may be, apart from the detection of the galacto waldenase system in mammary tissue, experimental evidence in its favour is lacking Reithel, Horowitz, Davidson and Kittinger (1952), have synthesized $\beta$ galactose 1 phosphate

but could demonstrate no appreciable formation of lactose from it when added to cell free mammary gland homogenates

A further theoretical possibility possessing advantages which make it worthy of consideration is that the condensation might involve phosphate esters of both glucose and galactose giving rise to a lactose phosphate which undergoing irreversible dephosphorylation as a final step would be continuously removed from the reaction thus shifting the equilibrium over towards synthesis In this connection it is interesting to note that McGeown and Malpress (1952) have detected by chromatographic methods, the presence of a lactose phosphate in milk

Before leaving the subject of lactose it is worth while to mention yet another speculation about its mode of synthesis in the mammary gland which has been put forward by Engelhardt (1950) Doudoroff and O Neal (1945) described a reversible reaction, catalyzed by an enzyme isolated from *B subtilis*, between glucose and a polysaccharide levan which is built up of fructose residues This reaction which in one direction gives a synthesis of levan, in the reverse direction yields sucrose It is essentially a trans glycosidation reaction Engelhardt s interesting speculations concern the possibility that lactose might be formed by an analogous trans glycosidation reaction between glucose and a polysaccharide built up of galactose residues A polysaccharide composed of galactose residues galactogen has been known for a long time but until recently had only been found in the garden snail It bears some resemblance to glycogen but on hydrolysis yields only galactose which be it noted consists almost exclusively of the $\beta$ epimer the form which exists in lactose The relevance of galactogen to the problem of the biosynthesis of lactose would be small were it not for the fact that in 1947 a galactogen differing slightly in properties from the galactogen of the snail was isolated from lung tissue of the bull (Wolfrom Weisblat Karabinos and Keller 1947) Since galactogen has now been found in animal tissues the question whether it occurs in the mammary gland assumes some importance As far as the writer knows no one has ever looked for galactogen in mammary tissue but it would seem to be worth while to examine this tissue for galactogen If it were found there the

possibility that lactose could arise by a trans glycosidation reaction analogous to the levan synthesis, as formulated in Figure VI 3, would at once arise Galactogen would presuma

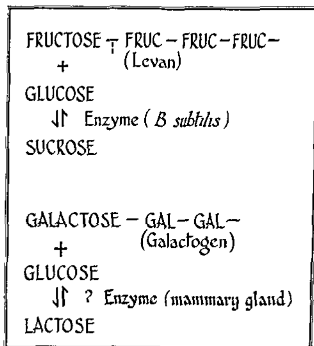



Fig VI 3

bly be formed from galactose 1 phosphate by the reversal of phosphorolysis but here again we encounter the problem of the formation of the $\beta$ galactosidic linkage

THE BIOSYNTHESIS OF MILK PROTEINS

Let us turn now to consider briefly some recent work on the biosynthesis of the milk proteins The proteins of milk consist principally of the phosphoprotein casein which occurs nowhere but in milk together with relatively minor amounts of the whey proteins which can be considered as consisting of two main fractions, lactalbumin and lactoglobulin All three fractions are probably complex mixtures of proteins Since the mammary gland is a site of active synthesis of protein it is a suitable organ for studying the mechanism of protein synthesis

and we may anticipate that useful advances in this difficult field may well result from current studies on the mammary gland involving the use of labelled amino acids

There seem to be four main possibilities to consider in respect of the mechanism of the biosynthesis of milk proteins Milk protein may be synthesized entirely from the amino acids of the circulating blood or partly from these and partly from amino acids arising from the degradation of blood plasma protein in the mammary gland Alternatively the milk proteins might arise from blood plasma proteins by rearrangement of peptide chains involving transpeptidation reactions or they may be derived only partly in this manner and partly from blood amino acids

Arterio venous studies carried out on the udders of lactating cows and goats have uniformly shown that there is an arterio venous difference in plasma amino acids across the lactating udder (Graham 1937 Shaw and Petersen 1938 Reineke *et al* 1939) The uptake is, however, small and it has been claimed, particularly by Shaw and Petersen (1938) that quantitatively it is insufficient to account for more than 40–50 per cent of the casein nitrogen of the milk

It was principally because of the general acceptance of this latter belief that attention was directed to the possibility that some of the plasma proteins might be used by the udder for the synthesis of milk protein Arterio venous studies carried out by Reineke *et al* (1941) led them to the conclusion that some fraction of the plasma protein probably a globulin fraction containing protein bound carbohydrate was utilized by the udder for the formation of milk protein However if plasma proteins are the sole source of the milk proteins the question arises whether the rate of regeneration of whatever fractions of the plasma protein are involved is sufficient to make good the appreciable drain on them due to the secretion from the body of considerable quantities of milk protein Such evidence as is available renders it doubtful whether this can be so

Recent work has directed attention once more to the possibility that a substantial proportion of the milk protein if not all of it is derived from the blood amino acids In the first place Bouckaert Oyaert Peeters and Sierens (1953) have

made interesting studies of the utilization of amino acids by the perfused isolated udder of the cow Since in the course of a perfusion lasting about two hours the blood would be recircul ated through the udder at least ten times, they reasoned that if milk proteins are formed from blood amino acids it should be possible to detect a significant decrease in the amino acid content of the perfusion blood during the course of the perfu sion Using microbiological methods for the determination of various free amino acids they found considerable differences in the concentration of ten amino acids in the perfusion blood between the beginning and end of the perfusion (see Table VI 1) The uptakes were usually most marked in the case of

TABLE VI 1

*Uptake of nine amino acids by the perfused, isolated bovine udder*

(from Bouckaert Oyaert Peeters and Sierens 1953)

| Amino acid | Conc at start of perfusion µg/ml | Left udder half no addition to blood | | Right udder half 15 g casaminoacids added to blood | |
|---|---|---|---|---|---|
| | | Conc at end of perfusion µg/ml | Uptake during perfusion mg | Conc at end of perfusion µg/ml | Uptake during perfusion mg |
| Histidine | 25 2 | 18 6 | 67 2 | 42 | 64 |
| Isoleucine | 12 3 | – | 96 | 32 4 | 410 |
| Leucine | 19 5 | 7 8 | 96 | 60 | 448 |
| Lysine | 14 9 | 5 3 | 80 | 17 3 | 544 |
| Methionine | 6 3 | 4 5 | 16 | 24 6 | 85 |
| Phenylalanine | 10 9 | 10 5 | 3 2 | 10 2 | 80 |
| Threonine | 9 3 | 6 3 | 24 | 38 1 | 116 |
| Tryptophane | 7 2 | 6 | 8 | 13 8 | 144 |
| Valine | 34 5 | 20 1 | 120 | 75 | 336 |

those amino acids which occur in the greatest concentration in the casein molecule It therefore seems reasonable to con clude that the disappearance of these amino acids during the perfusion must have been due to their absorption by the udder This view is supported by the fact that the amounts of the various amino acids absorbed by the udder during the per

fusion were considerably increased by the addition of a casein hydrolysate to the perfusion blood This work appears to demonstrate that there is a selective uptake by the udder of those amino acids which occur in the greatest amounts in casein and provides good evidence that blood amino acids must be reckoned as major precursors of milk protein

Recent studies with labelled amino acids have also led to a similar conclusion Barry (1952*b*) injected [*carboxy* $^{14}$C] lysine and [*carboxy* $^{14}$C] tyrosine into a lactating goat and measured the specific radioactivities of these two amino acids isolated from the casein and from the plasma proteins at various times after the injection He could thus compare their decay curves with those depicting the specific radioactivities of the free lysine and tyrosine of the blood plasma The curves for lysine, taken from Barry s paper, are shown in Figure VI 4

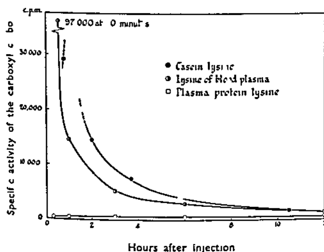



Fig VI 4 Radioactivity of the free lysine of plasma plasma protein lysine and of the casein lysine after an injection of C-lysine in a lactating goat *(from Barry 1952)*

It will be seen that the lysine isolated from the casein of the milk was, for some hours following the injection very much more radioactive than the lysine of the plasma protein More

over, the curves show that the radioactivity of the lysine of the milk casein at a given time was equal to that of the free lysine of the blood plasma about 1½ hours earlier This suggests that about 1½ hours before a given casein molecule was secreted in the milk all its lysine had existed as free lysine in the blood plasma The curves for tyrosine behaved similarly to those for lysine and it seems reasonable to conclude from these findings that all the lysine and tyrosine of casein arises from the free lysine and tyrosine of the blood at least in the goat Taken in conjunction with the work of Peeters and his colleagues on the perfused bovine udder, Barry s results suggest that all the essential amino acids of casein are derived from the free amino acids of the blood plasma

Essentially similar results for glycine, valine and lysine, all labelled with $^{14}C$ have been obtained by Campbell and Work (1952) in experiments on lactating rabbits Here again the radioactivity of the milk proteins, following the injection of the labelled amino acid was much higher than that of the blood plasma proteins They further showed that when labelled plasma proteins obtained by injection of $^{14}C$ glycine into a rabbit were infused into a second (lactating) rabbit, the proteins isolated from its milk were not appreciably labelled which also suggests that plasma proteins are not important precursors of milk proteins That the proteins of milk may not be derived wholly from plasma amino acids was, however, indicated by the fact that after injection of $^{14}C$ glycine, $^{14}C$-valine and $^{14}C$-lysine, these acids could be isolated with significantly higher specific activities from casein than from the whey proteins This suggested that the whey proteins might be derived partly from peptide fragments of plasma proteins a suggestion which is in harmony with the detection by Morton (1950) of a transpeptidase in cow udder tissue However, it is equally possible, as Campbell and Work point out, to explain these results on the assumption that the whey proteins are synthesized mainly from the plasma amino acid pool, but also, to a small extent arise from plasma protein In support of this interpretation was the later finding by Askonas, Campbell, Humphrey and Work (1954) that immune globulin of rabbit milk passes apparently unchanged from blood to milk, a finding which confirmed the suggestion made

long ago by Crowther and Raistrick (1916) Askonas Campbell and Work (1954) subsequently fractionated the whey proteins of the milk of a goat injected with radioactive glycine valine and lysine and found that, in the case of each acid, its specific activity at a given time after the injection was the same for casein as for $\beta$ lactoglobulin indicating that both proteins were synthesized in the mammary gland from the same amino acid pool This finding, however, does not altogether preclude the possibility that pre formed peptides were used to an equal extent in the synthesis of these two proteins That this is improbable is shown by a further series of experiments in which Askonas Campbell Godin and Work (1955) found that the $^{14}C$ labelled peptides obtained by partial hydrolysis of the casein and $\beta$ lactoglobulin isolated from goats milk after injection of radioactive glycine valine and lysine contained these amino acids labelled to the same extent as in the whole protein This uniform labelling of the amino acids throughout the protein chain suggests that casein and $\beta$ lactoglobulin are synthesized wholly from free amino acids of the blood

In conclusion it is worth while considering briefly what is known about the origin of the protein bound phosphorus which is present in the casein molecule in the form of phosphoserine The researches of Aten and Hevesy (1938) Barry (1952*b*) and of Colas, Le Bars Simonnet and Sternberg (1950), in which the radioactivities of various fractions of the milk phosphorus were studied after the injection of radioactive inorganic phosphate into lactating cows and goats leaves little room for doubt that the precursor of the casein phosphorus is the inorganic phosphate of the blood Simonnet and his colleagues however showed that radioactive inorganic phosphate continued to be secreted in the milk long after the casein had ceased to be radioactive This led them to put forward the view that phosphoserine is formed from a precursor which is stored in the mammary gland They suggest that this precursor may be phosphopyruvate from which phosphoserine could be formed by transamination

It may be expected that further work on the mode of formation of milk proteins which is in progress in various laboratories may eventually provide evidence bearing on the

rival theories of the mechanism of protein synthesis which are at present being debated The question is whether protein synthesis takes place by the simultaneous condensation of amino acids at a "template composed of nucleic acids, as suggested by Dounce (1952), or whether the synthesis involves the formation of peptide chains as intermediates as postulated by those who support the transpeptidation theory Campbell and Work (1953) discussed the possible bearing of some of their results on protein synthesis in the mammary gland on the idea that transpeptidation can occur in this organ Such an idea would be consistent with the above mentioned work of Morton (1950) and also with the results of Greenbaum and Greenwood (1954) who have shown that the cathepsin activity of rat mammary tissue increases markedly during lactation It is possible that intra cellular cathepsins are catalysts involved in transpeptidation

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